DEVELOPMENT OF AN INTERLEUKIN 2 RECEPTOR TARGETED GENE THERAPY VEHICLE

A Dissertation

by

WANIDA WATTANAKAROON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2005

Major Subject: Chemical Engineering

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ABSTRACT

Development of an Interleukin 2 Receptor Targeted Gene Therapy Vehicle.

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The effectiveness of most chemotherapeutic regimens is limited by the toxicity of the therapy to normal healthy cells. Therapies to selectively modulate abnormal T cells bearing the interleukin 2 receptor (IL-2R) have been developed to treat diseases associated with aberrant immune response. This study describes the development and optimization of a targeted gene or oligonucleotide therapy vehicle to IL-2R bearing T cells for selective elimination of these cells. In this work, a monoclonal antibody to the IL-2R was used to target the oligonucleotide delivery vehicle which consisted of a polyamidoamine dendrimer. Optimization of the delivery vehicle involves understanding the factors that govern its association with oligonucleotide, the pathway of IL-2R endocytic trafficking, and the stability of the oligonucleotide in the biological milieu. Oligonucleotide stability in a cellular environment was examined intra- and extracellularly. Results showed that the rate of intracellular degradation of oligonucleotides was much greater than extracellular degradation. Binding of oligonucleotides to dendrimers was demonstrated as a function of dendrimer generation. The total binding capacities for dendrimers differed depending upon dendrimer size and surface group, whereas equilibrium binding affinity was comparable for all dendrimers tested. Binding of oligonucleotide delivery vehicle to the cell surface and subsequent internalization was inversely related to dendrimer size, and in all cases, significantly less than binding and internalization of the natural ligand for the IL-2R. Based on experimental results, a kinetic model of the delivery vehicle was derived which included

the dependence of binding and internalization on dendrimer size and surface charge and intracellular degradation of oligonucleotide. Based on model predictions, we show that larger dendrimers carry more oligonucleotide than the smaller dendrimer vehicles, and delivery is more effective with larger vehicles. This work establishes our ability to predict the effects of different delivery vehicle properties on oligonucleotide delivery and aids in the development of design criteria for new vehicles for delivery of antisense, siRNA, or genes to IL-2R bearing cells.

DEDICATION

To the memory of my grandfather

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CHAPTER I
INTRODUCTION

Molecular level interventions are being developed in the field of medicine to facilitate advanced health care treatments. Immunomodulatory therapy, or the deliberate modification of the normal immune mechanism, holds great promise as a potential tool in the treatment of T cell cancers, autoimmune disease, and organ transplantation. Cancers of the T cells such as leukemia and lymphoma affect 100,000 people annually (The Leukemia & Lymphoma Society, 2004). Furthermore, it is estimated that approximately 70.4 million patients in the United States suffer from autoimmune related disease (Arthritis Foundation, 2002; National Multiple Sclerosis Society, 2003-2004). Current nonspecific therapies involving the use of immunosuppressive drugs for autoimmune diseases have significant side effects. The immune response associated with transplanted tissue antigens limits the success of organ transplantation in routine medical treatment. Therapies to specifically modulate T cell activity can be developed to treat a variety of cancers and autoimmune diseases and prolong graft survival.

In this project, we set out to develop the engineering or rational design of agents which deliver oligonucleotides to specific T cells to selectively and effectively modulate immune response (in this case to selectively kill one class of T cell). The plan of experimentation and modeling that follows describes the development and engineering of such agents. Achievement of these aims could be used to aid in the new avenues to treat diseases associated with aberrant immune response.

In chapter II, general background information on the immune system and current research regarding receptor targeted treatment are reviewed. This includes the introduction of the immune system, role of T cells and its cytokine receptors in health, the role of the lymphokine interleukin 2 (IL-2) and its receptor (IL-2R) in diseases, and novel perspectives for their treatment. For successful development of a more effective gene delivery agent, a minimal requirement typically involves an understanding of its

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This dissertation follows the style of Biotechnology and Bioengineering.

association with gene (i.e. binding affinity), uptake and transport (i.e. binding to a cell-specific receptor, internalization, subcellular trafficking, organelle escape, and nuclear translocation) into cells, and stability in the biological milieu. A number of components as followed are thus considered in designing and/or modulating a systemic *in vitro* gene delivery vehicle. In chapter III, the feasibility of using antisense oligonucleotide gene therapy as an anti-cancer treatment is assessed. We test the functional efficacies of selected oligonucleotides based on targeting with monoclonal antibody against the IL-2R expressed on leukemic cells. In addition, a high performance liquid chromatography (HPLC) technique is employed to explore the rate of degradation of oligonucleotides by serum and cytoplasmic nucleases. The ability of the delivery vehicle to protect the oligonucleotide against nuclease degradation is carefully examined. In chapters IV and V, we perform a kinetic analysis of the binding affinity and cellular uptake of the vehicle for oligonucleotide delivery. These data can provide new insights on the interrelationship between vehicle properties (size and charge) and efficacy of steps in the mechanism underlying gene delivery.

Having considered various design components, in chapter VI, we propose a mathematical model to yield insights into parameters for receptor-mediated gene delivery and/or potential therapeutic strategies to treat diseases. Mathematical models provide a means by which different parameters can be altered and can be potentially applied to predict experimental data. Finally, in chapter VII, conclusions from this work are drawn and outlines of possible future directions are presented.

CHAPTER II
IMMUNE SYSTEM IN HEALTH AND DISEASE, IL-2/IL-2 RECEPTOR T CELL SYSTEM, IMMUNOTHERAPY, AND ANTISENSE THERAPY AND OLIGONUCLEOTIDE DELIVERY

IMMUNE SYSTEM IN HEALTH AND DISEASE

The immune system is a complex network of cells that work coordinately to destroy any invader that compromises the health of the host. Dsyfunction or deficiency of components of this complex network can lead to a variety of clinical diseases. The immune response begins when the foreign antigens invade and trigger a series of events including T lymphocyte recognition, activation, and antigenic elimination. Following recognition and activation, antigen receptors on the T helper (T_H) cell surface bind to processed antigens positioned in the groove of major histocompatibility complex (MHC) class II molecules present on the surface of antigen-presenting cells (APC), leading to the release of IL-2 and interferon γ (IFN-γ). During the interaction between the T_H cell and the APC, interleukin 1 (IL-1) is released by the APC to promote IL-2 and IFN-γ secretion. Secreted IL-2 then feeds back to stimulate the expression of IL-2R on the surface of T_H cells as well as the production of various cytokines. The binding of secreted IL-2 to receptor-positive T cells induces clonal T cell proliferation. Activated T_H cells subsequently trigger the activation of T cytotoxic (T_C) cells to mediate cellular cytotoxicity and proliferation and differentiation of B cells that mediate the antibody-mediated response (Goldsby et al., 2000; Shames and Kishiyama, 2000). Figure 2.1 shows the activation of T_H cells and induction of the immune system (Goldsby et al., 2000). The cascade of reactions decribed lead the immune response attributes of specificity and memory.

The proper function of all components of the immune system is essential for a healthy immune response. A number of disease states occur as a result of the malfunction or imbalance of one or more immune system components.

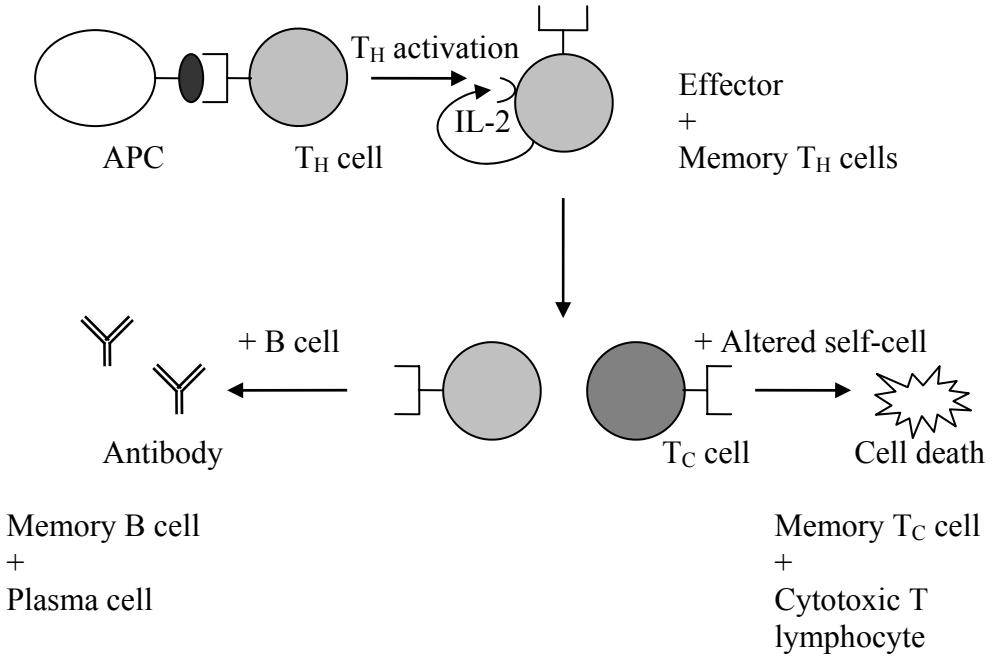


Figure 2.1. Activation of T_H cells and induction of the immune system.

Organ Transplantation

Transplantation is the act of transferring cells, tissues, or organs from one site to another. T cells from the transplanted organ attack the transplant recipient, resulting in graft versus host diseases (GVHD). Conversely, T cells from the host can attack the new organ, resulting in graft rejection.

Graft rejection is mainly caused by a T cell-mediated immune response to alloantigens expressed on cells of the foreign graft. Graft versus host disease is somewhat analogous, except that activated T cells from the graft trigger an immune response against antigens on the host cells. Once activated, $CD4^+$ T cells secrete cytokines and induce a number of responses from other effector cells. Many cytokines direct alloresponsive T cells to develop an allospecific immune response, leading ultimately to either transplant tolerance or rejection. Proinflammatory cytokines such as interleukin 12 (IL-12) and interleukin 15 (IL-15) appear to promote graft rejection through directing T_H cell differentiation or promoting expansion, survival, and memory development, respectively. On the other hand, immunosuppressive cytokines such as interleukin 10 (IL-10) and transforming growth factor β (TGF-β) may induce host tolerance through mediating regulatory T cell homeostasis and function (reviewed in Walsh et al., 2004). Soulillou (1999) has shown that anti-IL-2R antibodies against the IL-2 high affinity receptor in allorecognition efficiently decreased transplantation rejection. In addition, other growth factors such as interleukin 4 (IL-4), interleukin 7 (IL-7), and IL-15 may be involved in this phenomenon. Currently, immunosuppressive therapy is used during tissue transplantation. During tissue transplantation, drugs are administered which block the formation of cytokines or their receptors which would aid in the mounting of an immune response against the foreign tissue. Clinically, mitotic inhibitors, corticosteroids, cyclosporin A, tacrolimus, FK506, rapamycin, and x-irradiation have been given to transplant recipients with harmful side effects (Goldsby et al., 2000; reviewed in Walsh et al., 2004).

During transplantation, the immune system is nonspecifically suppressed. Prolonged use of immunosuppressive drugs is associated with high incidences of

infection and cancers. The development of an immunomodulatory strategy that specifically suppresses immune response to the donated tissue could significantly improve the likelihood of success and function of the donated organ without complications associated with the nonspecific immunosuppressive therapies.

Autoimmune Diseases

Autoimmune disease, a group of disorders that involve tissue injury or pathology, is caused by the activation of autoreactive T cells and/or B cells to self-antigens, or autoantigens (reviewed in Davidson and Diamond, 2001; Lernmark, 2001; Christen and von Herrath, 2004). This activation can cause cytolysis and tissue destruction in the host organism.

An autoimmune disease is initiated by response to a single antigen. Later in the disease, the evolution of the response causing autoreactivity, referred to as epitope spreading, may involve a change in different effector cells and inflammatory mediators as the disease progresses (reviewed in Davidson and Diamond, 2001). Furthermore, an increase in susceptibility to autoimmunity is controlled by the genes of the individual and the environment (reviewed in Marrack et al., 2001). Christen and von Herrath (2004) reported that autoimmune initiation includes the genetic predisposition, naïve lymphocytes that react with autoantigens, and a precipitating event that leads to cell activation. Because autoantigens have been recognized by the autoantibodies associated with autoimmune disorders, detection of autoantibodies might help in the development of clinical approaches to predict disease (Lernmark, 2001).

Approaches to the treatment of autoimmune diseases are analogous to treatment of GVHD or transplant rejection. Treatments range from nonspecific immunosuppressive regimens to more specific molocular therapies. Activated T cells, expressing the high affinity IL-2R and some co-stimulatory signaling molecules on the surface, have been used as targets for treating autoimmune disease (Kremer et al., 2003; Swiatecka-Urban, 2003; Nickoloff and Nestle, 2004). Several studies have shown that

selective removal of all $CD4^+$ cells improves symptoms associated with experimental autoimmune diseases (Onodera et al., 2003; Nakane et al., 2003; Griffiths, 2004).

Examples of Known Organ-Specific and Systemic Autoimmune Diseases

Systemic Lupus Erythematosus. Systemic lupus erythematosus (SLE), referred to as lupus, is a systemic autoimmune rheumatic disease that produces multiple autoantibodies (i.e. antinuclear and anti-DNA antibodies) to various tissue antigens expressed throughout the host, leading to variable symptoms dependent on the affected individual (Goldsby et al., 2000). Pathologically, the autoantibodies bind to extracellular molecules in the target organs and activate the inflammatory effector functions at that site, resulting in tissue damage. Then, the development and propagation of SLE are further caused by impairment of normal tissue clearance of those damaged or apoptotic cells in tissues (Greidinger and Rosen, 2000).

Multiple Sclerosis. Multiple sclerosis (MS) is a primary demyelinating disease of the central nervous system of unknown etiology. Approximately 400,000 Americans acknowledge having MS, and the average person has a one in 1,000 chance of developing the disease (National Multiple Sclerosis Society, 2003-2004). It is generally believed that autoreactive T cells contribute significantly to the demyelinating process. There are also some suggestions of the cause of MS in certain virus infection (Goldsby et al., 2000).

A variety of immunosuppressive therapies have been tested to date with varying degrees of success. There is a hope that specific elimination of activated T cells would serve as a potential improvement in the treatment of MS.

Rheumatoid Arthritis. Rheumatoid arthritis (RA) is a common autoimmune disorder that produces a group of autoantibodies called rheumatoid factors. This disease is characterized by a chronic synovial inflammation of the joints, followed by tissue destruction at the cartilage junctions. This leads to deformities and loss of articular function. The classic rheumatoid factor is an IgM antibody which can form complexes with normal circulating IgG, as an autoantigen, and are deposited in the joints. These

complexes then activate the complement system, leading to chronic inflammation of the joints (Goldsby et al., 2000).

Scleroderma. Scleroderma is a chronic degenerative, but rare, disease that affects the skin, internal organs and the vasculature, leading to hardening and tightening of the skin and connective tissues. Scleroderma results from the overproduction and accumulation of collagen in connective tissue. There are also some suggestions of the cause of scleroderma in fetal cells' immune response (Goldsby et al., 2000; Mayo Foundation for Medical Education and Research, 2004).

Neoplasia and Cancer

T cell cancers involve the uncontrolled proliferation of a clonal population of T cells. Homeostasis in normal tissue is balanced by a process of cellular proliferation and cell death regulated by oncogenes and tumor suppressor genes, respectively. It is believed that genetic alterations underlie all cellular and biochemical aberrations responsible for the malignant phenotype in human cancer. Gene alterations include serial oncogene activation and tumor suppressor gene inactivation (Tripathy, 2000).

The leukemias and lymphomas are malignant tumors or cancers of hematopoietic cells of the bone marrow. T-cell leukemias and lymphomas involve a proto-oncogene that has been translocated into the T-cell-receptor genes. Leukemias proliferate as single cells in circulation, whereas lymphomas, Hodgkin's and non-Hodgkin's, grow as solid tumors within a lymphoid tissue. Leukemias are classified as acute or chronic according to the progression of the disease and cell maturity involved (Goldsby et al., 2000). Non-Hodgkin's lymphoma is less predictable than the Hodgkin's lymphoma. Non-Hodgkin's lymphoma is particularly aggressive in HIV-infected patients (Shames and Kishiyama, 2000).

The inducible IL-2R α chain is a component of the high affinity trimeric receptor. It is not expressed on resting cells. However, it is expressed on the surface of abnormal cells in a number of neoplasms of the immune system (Waldmann, 1993).

While there are many more diseases of the immune system, the ones described all share in common the involvement of T cells and the IL-2R in the disease, either at an inappropriate level or as the marker of cell activation. Understanding the involvement of the IL-2/IL-2R system in disease provides a framework for the development of molecular level therapies to diseases involving T cells.

IL-2/IL-2 RECEPTOR T CELL SYSTEM

The T cell is one of the components of the immune system. T cell activation begins when the processed and presented foreign protein antigens interact with antigen-binding receptors on T_H cells. This interaction generates a signal along with an essential co-stimulatory signal that up-regulates the expression of the IL-2 and its high affinity receptor (IL-2R α chain), leading to proliferation and differentiation of the T_H cells. The antigen-specific activated T_H cells secrete various cytokines, as a result of cell-specific interaction, that induce a specific immunologic response including the differentiation of B cells (IL-4, IL-5, IL-6, and IL-10) and the activation of T_C cells (IL-2, IFN-γ, and TNF-β) and macrophages (Goldsby et al., 2000). The cytokines have been sited as important regulators in T-cell function (Powrie and Coffman, 1993). Because of its role in the regulation/modulation of immune response, the control of T_H cells and the development of immunomodulatory therapies targeting IL-2/IL-2R proved useful in the treatment and prevention of human diseases.

The T cell receptors are expressed as either the $\alpha\beta$ or $\gamma\delta$ heterodimers (Lefranc and Lefranc, 2001). The α-subunit contributes to binding of IL-2 and upregulation of receptor sensitivity, whereas the β- and γ-subunits contribute mainly to receptor-mediated internalization and signal transduction (Takeshita et al., 1992; Morris and Waldmann, 2000). Indeed, IL-2 might initially interact with the β chain constitutively present on resting T cells. This binding leads to the rapid induction of genes (i.e. *c-myc* and *c-myb*) involved in T cell activation and to the expression of genes required for T cell progression including Tac forming the high affinity IL-2R (Bich-Thuy et al., 1987).

Subtil et al. (1998) detected the β cytosolic domain expressing a unique sorting signal involved in targeting the receptor toward degradation.

The high affinity IL-2R is highly expressed on activated T cells. It is a heterotrimer consisted of three distinct membrane components, IL-2R α chain (Tac, CD25, p55, 55 kDa), β chain (CD122, p70 or p75, 70-75 kDa), and γc chain (CD132, p64, 64 kDa), associated in a noncovalent manner. A schematic representation of the high affinity IL-2R is given in Figure 2.2 (Minami et al., 1993). The β and γc chains, the chains associate, form the intermediate affinity receptor that is present on resting T cells. A comparison of the subunits of the IL-2R forms is provided in Table 2.1 (Goldsby et al., 2000). Indeed, normal cells do not express the IL-2R α in a resting state (Waldmann, 1993). Expression of the α-subunit of the high affinity receptor thus plays an important role in T-cell activation and is of clinical importance. A number of IL-2R α related diseases are listed in Table 2.2 (Morris and Waldmann, 2000).

TARGETED DRUG DELIVERY USING IL-2/IL-2 RECEPTOR

For over 20 years, researchers have been actively investigating means to selectively treat diseased cell such as cancers as a means of increasing the therapeutic dose delivered to the diseased tissue while minimizing side effects of surrounding healthy tissue. IL-2R expressing abnormal T cells, either cancerous or inappropriately stimulated, have been targeted via either the IL-2 ligand or the anti-IL-2R antibody for selective elimination of these cells. Such strategies have been the focus of therapeutic intervention associated with lymphoid malignancies, select autoimmune diseases, GVHD, and allograft rejection.

The chimeric or humanized antibodies selected for antibody-targeted therapy have increased their clinical usefulness in the treatment of leukemia and lymphoma with a favorable pharmacokinetic profile and low immunogenicity (reviewed in van de Loosdrecht et al., 2004). Anti-IL-2R antibodies targeting the IL-2R approved by the US Food and Drug Administration are listed in Table 2.3 (reviewed in Waldmann, 2003). Strategies targeting the IL-2R with anti-IL-2R antibodies to inhibit its proliferation by

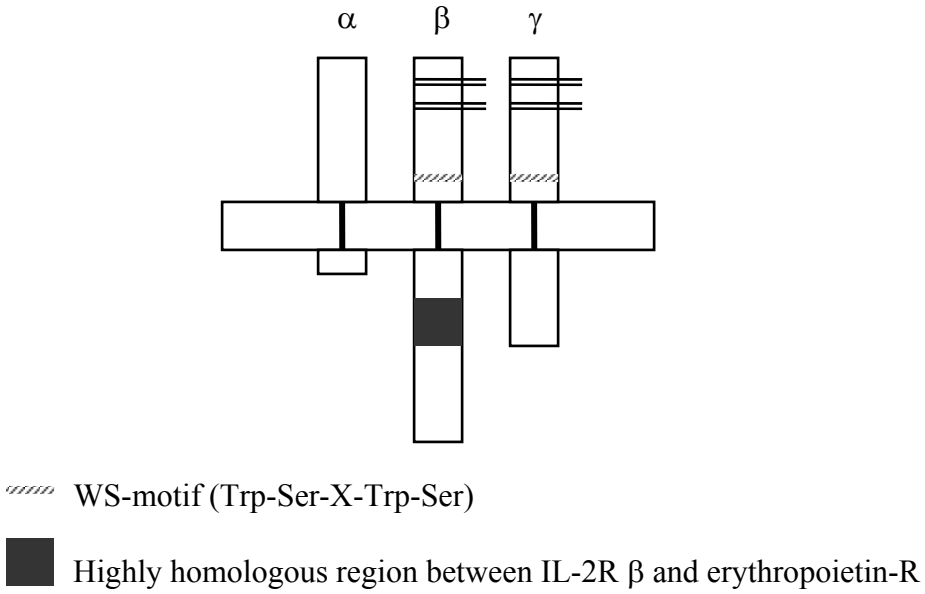


Figure 2.2. Schematic representation of IL-2 receptor (Reprinted, with permission, from the *Annual Review of Immunology*, Volume 11 © 1993 by Annual Reviews www.annualreviews.org).

Table 2.1. Comparison of the subunits of three IL-2 receptor forms. The overview on association and dissociation constants is also indicated (For a line drawing from KUBY IMMUNOLOGY, 4/e by Richard Goldsby, et.al. © 1992, 1994, 1997, 2000 by W. H. Freeman and Company. Used with permission).

	Intermediate affinity IL-2R βγ	**High affinity IL-2R αβγ**	**Low affinity IL-2R α**
Subunit composition:	IL-2R β IL-2R γ	IL-2R α IL-2R β IL-2R γ	IL-2R α
Affinity constant (K_a):	10^7 M	10^{11} M	10^8 M
Dissociation constant (K_d):	10^{-9} M	10^{-11} M	10^{-8} M

Table 2.2. A listing of some IL-2R α related diseases (Annals of the Rheumatic Diseases, 2000, Vol 59, pages i109-i114, reproduced with permission from the BMJ Publishing Group).

Allograft rejection
Bone marrow
Cardiac
Liver
Renal

Autoimmune disease
Aplastic anaemia
Behcet's syndrome
Crohn's disease
Giant cell arteritis
Juvenile rheumatoid arthritis
Kawasaki disease
Multiple sclerosis
Polymalgia rheumatica
Rheumatoid arthritis
Sarcoidosis
Scleroderma
Sjögren's syndrome
Systemic lupus erythematosus
Vasculitis
Wegerner's granulomatosis

Neoplasia
Acute myelocytic leukaemia
Anaplastic large cell lymphoma
Adult T cell leukaemia/lymphoma
Chronic lymphocytic leukaemia
Chronic myelocytic leukaemia
Cutaneous T cell lymphoma
Mycosis fungoides
Hairy cell leukaemia
Hodgkin's disease
Non-Hodgkin's lymphomas (B cell)
Peripheral T cell lymphomas

Table 2.3. A listing of anti-IL-2R antibodies approved by the US Food and Drug Administration (Reprinted with permission from Nature Medicine, Waldmann TA. Copyright 2003 by Nature Publishing Group).

Product	**Type**	**Target of action/Condition**	**Approved**
Daclizumab (Zenapax)	Humanized	CD25 (IL-2R α, Tac) on activated lymphocytes/ Transplant allograft rejection	1997
Basiliximab (Simulect)	Chimeric	CD25 (IL-2R α) on activated lymphocytes/ Transplant allograft rejection	1998

preventing IL-2 binding or using antibody-toxin or -radionuclide conjugates to kill targeted cells have been previously investigated (reviewed in Waldmann, 1989; Queen et al., 1989; Waldmann, 1991; Waldmann, 1993; Waldmann, 2000). Waldmann (1991) has proposed that the effective immunoconjugates must internalize into the endosome and further translocate to the cytoplasm, where the inhibition of protein synthesis occurs. Walz et al. (1990) have demonstrated that inhibition of gene and protein synthesis by IL-2/diphtheria toxin-related fusion protein targeting rIL-2 stimulated T cells appears to be dose dependent. With anti-Tac (anti-CD25), a p55 IL-2R α chain monoclonal antibody, the stimulatory effect of IL-2 toxin, under conditions that responded to high IL-2 toxin, was mediated by IL-2R binding domain of the fusion protein. Analogously, with both IL-2 toxin and IL-2, stimulation of *de novo* transcription of a heat shock protein gene in phytohemagglutinin (PHA) activated T blasts was shown to have a similar pattern. The γ- and β-emitting radionuclides linked to antibodies suitable for therapy include ^{213}Bi, ^{212}Bi, ^{212}Pb, ^{211}At, ^{90}Y, and ^{131}I. Suitable γ-emitting nuclides including ^{211}At, ^{212}Pb, and ^{212}Bi, which can release high energy emissions over a short distance, are efficient at killing the leukemic cells. A high energy emission assures the destruction of the cell, while protecting other nearby healthy cells. Particularly, the most successful radionuclides used in leukemia and lymphoma are the β particles emitted by ^{131}I and ^{90}Y. Given that radionuclides act over a short distance and do not need to enter target cells, the use of antibody-radionuclide conjugates is effective for their treatment of tumor cell antigenic heterogeneity (Waldmann, 2000). A ^{90}Y-IL-2R anti-Tac has been used clinically with some success for treatment of adult T-cell leukemia (Waldmann et al., 1995). Conjugates using IL-2 and a portion of the *Pseudomonas* exotoxin have been developed and were found to be effective in mouse models of tissue transplantation, tumors expressing the IL-2R, and autoimmune disease (Waldmann, 1993).

While there have been many successes with targeted therapies to IL-2R bearing cells, in general, internalization and intracellular degradation of the immunotoxin govern (and severely limit) the effectiveness of therapy (Press et al., 1988; Braslawsky et al., 1991; May et al., 1991; Yazdi et al., 1995).

ANTISENSE THERAPY AND OLIGONUCLEOTIDE DELIVERY

The antisense (anti-mRNA) therapeutic approach is the use of sequence-specific antisense oligonucleotides to interact with a complementary sequence on a targeted mRNA. This specific base pair interaction leads to the destruction of a targeted mRNA and the inhibition of transcription and translation of a specific protein (Gewirtz et al., 1996; Eichman et al., 2000; Phillips and Gyurko, 1997). Antisense techniques primarily include triple-helix formation with the DNA double helix and hybridization to the newly formed RNA to prevent its transcription. Inhibition of gene expression is mainly accomplished by activation of ribonuclease (RNase) H. RNase H is an endonuclease found both in the cytoplasm and the nucleus. RNase H cleaves the RNA strand of a DNA-RNA heteroduplex. Several cancer-relevant genes known to be important in the regulation of apoptosis, cell proliferation, metastasis, and angiogenesis have been validated as molecular targets for antisense therapy (see Table 2.4) (reviewed in Jansen and Zangemeister-Wittke, 2002). Antisense therapy could prove to be a useful strategy in modulating the function of IL-2R expressing T cells involved in a number of cancers, as well as autoimmune and graft versus host diseases.

Oligonucleotide transfer technology is designed to introduce genetic materials into abnormal cells to modulate the expression of a target protein. Steps involved in oligonucleotide transfer include cellular uptake, intracellular trafficking, and (if necessary) nuclear translocation. Ideally, the delivery system should be able to protect the oligonucleotide from extracellular degradation and to effect cell-specific delivery to a target cell. A number of investigators have explored the structure and transfection parameters of cationic lipids and cationic polymers as a means of increasing efficacy of oligonucleotide delivery (Safinya et al., 2002; Holtorf and Mikos, 2002).

In this work, targeted oligonucleotide delivery to IL-2R bearing cells is examined as a means of treating certain diseases including specifically a model T cell cancer. In order to increase the specificity and efficacy of oligonucleotide delivery, we have proposed to use a monoclonal antibody for targeting and a positively charged polyamidoamine dendrimer as the carrier for the oligonucleotide to the IL-2R bearing T

Table 2.4. A listing of antisense targets (Reprinted with permission from Elsevier (**Lancet Oncology**, 2002, Vol 3, pages 672-683)).

Cell cycle
Cyclin D
MYC
CDKs
Mitogenic pathways growth signalling
EGFR
PKA1
HER2/NEU
IGF1
IGFBP2
BRAF
PKCα
RAS/RAF
Impaired apoptosis
A1
MCL1
BCL-2
BXL-XL
BCL-W
IAPs
Mdm2
Clusterin
HSP70
MITF
Infinite lifespan
Telomerase
Angiogenesis
VEGF
VEGFR
bFGF
Metastasis
MMPs
Aberrant gene regulation
DNA methyltransferase
Histone deacetylase

Bold indicates mRNA target for antisense therapy in phase I-III clinical trials.

cells. We believe that the impact of the work presented is in the development of the systematic understanding of the role of vehicle properties in the effectiveness of delivery of oligonucleotide to cells. This work will enable the development of appropriate design strategies for effective oligonucleotide delivery. In addition, the work could aid development of a clinically useful treatment for autoreactive T cell diseases and be of use during tissue transplantation and T cell leukemias, where high expression of the IL-2R is present.

CHAPTER III
ANTISENSE OLIGONUCLEOTIDE AND RNA INTERFERENCE THERAPEUTIC INTERVENTION AND STABILITY

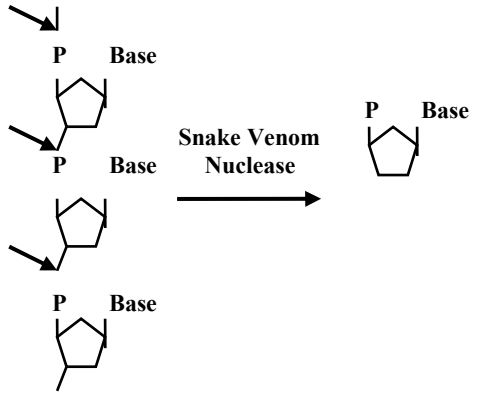
INTRODUCTION

The antisense (anti-mRNA) therapeutic approach is the use of sequence-specific antisense oligonucleotides (13-25 bases, single-stranded RNA or DNA molecules) to interact with complementary sequence on a targeted mRNA. This specific base pair interaction leads to the destruction of a targeted mRNA and the inhibition of transcription and translation of a specific protein (Gewirtz et al., 1996; Eichman et al., 2000; Phillips and Gyurko, 1997). Antisense techniques primarily include triple-helix formation with the DNA double helix and hybridization to the newly formed RNA to prevent its transcription.

Oligonucleotides, in their natural form, have a phosphodiester (PO-) backbone that is readily degraded by serum or cellular (exo- and endo-) nucleases (Phillips and Gyurko, 1997; Toth et al., 2002). A schematic of typical nucleases is provided in Figure 3.1 (Mishra, 2002). Phosphorothioate (PS-) analogs, containing a single sulfur atom replacing an oxygen atom in a nonbridging position with phosphorus, have become attractive as the modification of choice due to their affinity for targeted mRNA, greater resistance to nuclease degradation due to chiral internucleotide linkages, and activation of ribonuclease (RNase) H-mediated RNA degradation. However, the increased oligonucleotide-protein (i.e. nucleic acid polymerases) binding, polyanionic and immune stimulatory characteristics of PS-analog are believed to affect their specificity and mechanism of actions (Phillips and Gyurko, 1997; Roush, 1997; Agrawal, 1999).

RNA-mediated interference (RNAi) is emerging as a groundbreaking discovery in the field of nucleic acid-based gene control. RNAi relies on the *in vivo* generation of short interfering RNA (siRNA) as an effector of targeted gene silencing at the posttranscriptional level. Short interfering RNA is a double-stranded RNA fragment of approximately 21-23 base pairs produced from the long double-stranded (ds)RNA

A



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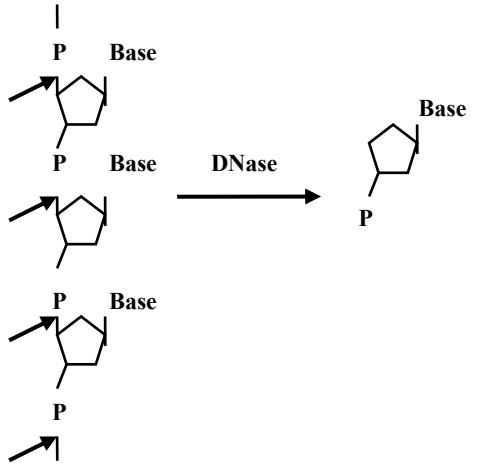

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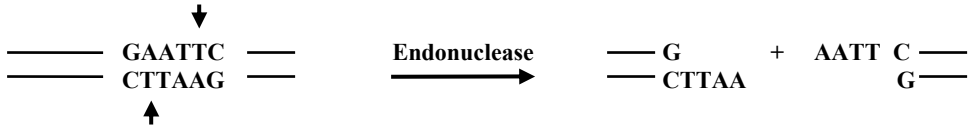


Figure 3.1. Schematic of nucleases. (A) Phosphodiesterase. (B) DNase. (C) Restriction endonuclease (Reprinted with permission of John Wiley & Sons, Inc from Nucleases: Molecular Biology and Applications, by Mishra NC, 2002, Wiley-Interscience, New Jersey. Copyright © 2002 by John Wiley & Sons, Inc).

molecule. In the siRNA pathway, long dsRNA is cleaved by the RNase III family member, called Dicer, into siRNAs in an ATP-dependent reaction. These siRNAs are then incorporated into the RNA-induced silencing complex (RISC), a second endoribonuclease complex. The duplex siRNA is unwound, leaving the encoded antisense strand to guide RISC to its complementary sequence of targeted mRNA for cleavage (Zamore, 2002; reviewed in Dykxhoorn et al., 2003). Recently, Gautherot and Sodoyer (2004) have proposed the multi-model strategy for design and optimization of siRNA sequences. Interestingly, siRNA has now been used as a potential therapeutic tool to target specific oncogenes (Damm-Welk et al., 2003) in mammalian cancer cell lines (Kittler and Buchholz, 2003). Furthermore, Bertrand et al. (2002) have reported that siRNA appears to be more efficient than antisense oligonucleotide, likely due to its higher resistance to nuclease degradation.

In the work described here, we examined the relative stability of oligonucleotides, both phosphodiester and phosphorothioate analogs, against intracellular and extracellular nucleases using a biological activity assay (toxicity) and an HPLC method. We describe the development of an HPLC method to measure the rate of oligonucleotide degradation in the presence and absence of dendrimer. We identify the rate of intracellular degradation to be much greater than extracellular degradation.

MATERIALS AND METHODS

Materials

Synthetic wholly PO- and PS-linked oligonucleotides molecular weights 5,485 and 5,773, respectively, were purchased from Gene Technologies Laboratory, Institute of Developmental and Molecular Biology, Texas A&M University (College Station, TX) and Sigma-Genosys (The Woodlands, TX). Commercially available PAMAM dendrimers were purchased from Aldrich (Milwaukee, WI) and used without further purification. Cell culture reagents were purchased from GibcoBRL (Grand Island, NY). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased

from Pierce (Rockford, IL). All other chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, MO).

Oligonucleotide

Single-stranded 18-bp PO- and PS-oligonucleotides to the 63-kDa form of phosphodiesterase 1 (PDE1B1) were synthesized (see Figure 3.2). The PS-oligonucleotide was modified at every base position. This oligonucleotide was targeted against the translation initiation region of the human B-lymphoblastoid cell line mRNA encoding PDE1B1, which is selectively expressed in leukemic and actively growing lymphocytes (Jiang et al., 1996). A sequence was manipulated and homology searches were performed against nucleic acid sequence database Biology Workbench Version 3.2, University of California San Diego, for the possibility of cross-reaction.

Oligonucleotide Solution Preparation

Stock solutions of 10-fold μM were prepared by dissolving the oligonucleotides in TE (10 mM Tris, 1 mM EDTA, pH 8.0). The stock solutions were diluted in phosphate buffer saline (PBS, 13.67 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2) to the concentrations used in the experiment.

Cell Culture

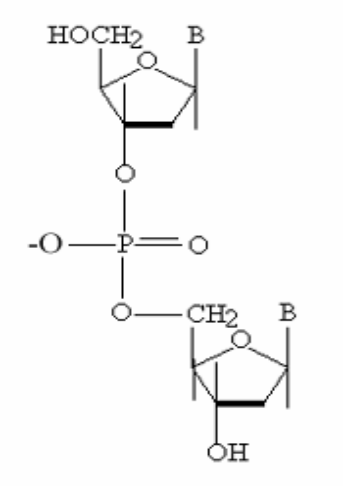
Mouse HB-8555 hybridoma cells (ATCC, Rockville, MD) were cultured in a humidified 5% (v/v) CO_2/air environment at 37°C in RPMI supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 10 mM HEPES, 4.5 g/L glucose, 1 mM sodium pyruvate, 0.025 mM 2-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (pH 7.4). Culture supernatant was collected every 4 to 5 days and stored at 4°C for further antibody purification. Human T-cell leukemia TIB-152 Jurkat cells (ATCC, Rockville, MD) were cultured in a humidified 5% (v/v) CO_2/air environment at 37°C in RPMI supplemented with 10% (v/v) FBS, 10 mM HEPES, 4.5 g/L glucose, 2 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and

Oligonucleotide

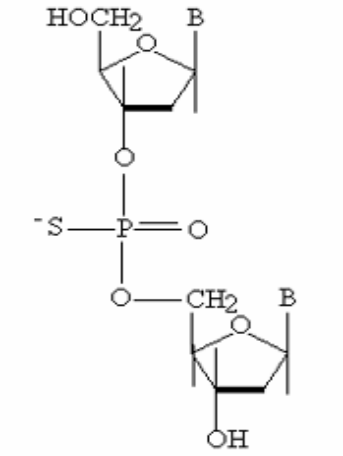
5' GGA CAG CTC CAT GCT CAG 3'

Oligonucleotide Structures

PO-



PS-



Target PDE1B1 Sequence

5' gctrgtccmygccagccgcagaccgtggctgagc ATG GAG CTG TCC CCC CGC … 3'
---------- untranslated region ---------- ---------- open reading frame ----------
r = A or G
m = A or C
y = C or T

Figure 3.2. Schematic of PO- and PS-oligonucleotide sequences used in the study.

2.5 μg/mL amphotericin B (pH 7.4). For the oligonucleotide toxicity experiments, cells were plated at densities ranging from 5 to 6 x 10^5 cells/well in 96 well plates.

IL-2 Receptor Monoclonal Antibody Purification

Anti IL-2R (CD25) monoclonal antibody was purified from cell culture supernatant by affinity chromatography using a protein A Sepharose 4 Fast Flow column (Pharmacia, Piscataway, NJ) via standard techniques (Harlow and Lane, 1988; Montage Antibody Purification Kit and Spin Columns with PROSEP-A Media User Guide, Millipore, Bedford, MA). The culture supernatant from HB-8555 cells was filtered through a 0.22-0.45 μm pore size, 47 mm diameter filter (Millipore, Bedford, MA) and diluted 1:1 (v/v) in a binding buffer (1.5 M glycine/NaOH, 3 M NaCl, pH 9.0) before loading. The column was washed with 10 bed volumes of binding buffer to remove unbound contaminants. The bound antibody was then eluted with an elution buffer (0.1 M sodium citrate, pH 5.5).

The pH of purified antibody was neutralized using a 1:20 (v/v) neutralization buffer (1M Tris/HCl, pH 9.0). The antibody solution was concentrated using the Microcon ultra centrifugal filter device with a 30000 molecular weight cut off membrane (Millipore, Bedford, MA). The concentration and purity of antibody were then determined by the absorbance reading at 280 nm (1 OD is approximately equal to 0.8 mg/mL) and SDS PAGE using the pHastgel system (Pharmacia, Piscataway, NJ), respectively. An antibody was stored at -20°C until use.

Antibody Dendrimer Conjugation

Solutions of dendrimer and EDC were mixed in a conjugation buffer (0.1 M MES, pH 5.0) in a molar ratio of 1:1. An antibody was added to the complex mixture and rotated at 60 revolutions per minute at room temperature for 3 hours. An antibody dendrimer conjugate was then purified on a PD-10 desalting column (Pharmacia, Piscataway, NJ) in phosphate buffer saline (PBS, 13.67 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2), and the first peak containing antibody was

collected. Unreacted reagents were further removed via ultra filtration through a 30000 molecular weight cut off membrane (Millipore, Bedford, MA). The protein content was then measured with the absorbance readings at 280 nm and the BCA assay (Walker, 2002) to calculate the degree of conjugation.

MTT Reduction Assay

Jurkat cell viability was measured using the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay (Pollack et al., 1995). The oligonucleotide samples with or without an antibody dendrimer conjugate were incubated with the cells for various lengths of time, after which time MTT was added to the culture medium to yield a final MTT concentration of 0.5 mg/mL. Cells were incubated with the MTT for 4 hours in a CO_2 incubator after which time 100 μL of a 5:2:3 N, N-dimethylformamide (DMF): sodium dodecyl sulfate (SDS): water solution (pH 4.7) was added to dissolve the formed formazan crystals. Then, after 20 hours of incubation in a humidified CO_2 incubator, the MTT absorbance was measured using an Emax Microplate reader at 585 nm (Molecular Devices, Sunnyvale, CA). Viability was reported relative to control cells unexposed to the oligonucleotides with or without an antibody dendrimer conjugate.

Conditioned Medium

Conditioned medium was taken from the 3-day Jurkat cells. Cells were removed by centrifugation (500 rpm, 8 min), and the resulting supernatant was sterile filtered using a 0.2 μm syringe filter unit (Nalge Nunc International Corporation, Rochester, NY). The total protein content was measured with the BCA assay (Walker, 2002).

Nuclease Containing Cellular Extract

Jurkat cells at a density of 8×10^6 cells/mL were collected by centrifugation (500 rpm, 8 min) and then thoroughly washed twice with cold phosphate buffer saline. Subsequently, they were resuspended in1.2 mL of low ionic-strength phosphate buffer (5

mM Na_2HPO_4, 0.88 mM KH_2PO_4, pH 7.2) and sonicated using an ultrasonic bath-cleanser (Cole-Parmer, Vernon Hills, IL) for 2 minutes. Cellular debris was then removed by centrifugation at 17400 x *g*, 4°C, 20 min. The total protein content in the cell lysate was measured with the BCA assay (Walker, 2002).

Oligonucleotide Degradation Assay

Stability was assayed by incubating 30 μM PO-oligonucleotides in phosphate buffer saline (PBS, 13.67 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2), RPMI containing 10% (v/v) heat-inactivated FBS, conditioned medium and the cell lysate at 37°C in a total volume of 100 μL in a microcentrifuge tube. After the specified time, the hydrolysis products were identified by an UltiMate™ capillary HPLC system with UV detection at 260 nm (LC Packings, BV, Dionex, SanFrancisco, CA) using a Superdex Peptide PC 3.2/30 (bed dimensions: 3.2 x 300 mm) gel filtration column (Pharmacia, Piscataway, NJ). HPLC conditions are given in the figure captions. The relative concentrations of full-length oligonucleotide or hydrolysis products were then quantified by the peak areas in the chromatograms. The peak area was calculated as the total sum of $\frac{mAU_1 - mAU}{t_1 - t} = \frac{mAU_1 - mAU_2}{t_1 - t_2}$.

Complex Degradation Assay

To evaluate the ability of the dendrimer to protect the oligonucleotide from degradation, the complex at a molar ratio (= molar fraction of oligonucleotide/molar fraction of dendrimer) equivalent to 1:3 was formed by mixing the oligonucleotide with the dendrimer at a concentration of 10 and 30 μM, respectively, in Tris buffer saline (TBS, 13.67 mM NaCl, 2.68 mM KCl, 25 mM Tris, pH 8.0). The complex mixture was then rotated at 60 revolutions per minute at room temperature for 2 hours. Stability was assayed by incubating the complexes in the cell lysate at 37°C in a total volume of 100 μL in a microcentrifuge tube. After the specified time, urea, n-octyl-β-D-glucopyronoside, or 1 N NaOH was gradually added in order to dissociate the

complexes. Gradient separation of oligonucleotides and dendrimers was performed using an UltiMate™ capillary HPLC system with UV detection at 215, 260, and 280 nm (LC Packings, BV, Dionex, SanFrancisco, CA) using a ProPac WCX-10G (bed dimensions: 4 x 50 mm) guard column (Dionex, Sunnyvale, CA). HPLC conditions are given in the figure captions. The first peak containing oligonucleotide was collected, and the fraction was then identified by HPLC using a Superdex Peptide PC 3.2/30 (bed dimensions: 3.2 x 300 mm) gel filtration column (Pharmacia, Piscataway, NJ).

Data Analysis

All data were presented as the mean ± standard deviation of n independent observations. To determine whether a treatment data set was significantly different than a control data set, the *p*-value was calculated by a two-sample, two-sided *t*-test comparing the mean to the mean of the control.

Model and Parameter Estimation

The degradation of oligonucleotide was assumed to be first order in oligonucleotide and protein (or nuclease) content of the medium. The following system of equations describes the mass action kinetics and exponential decay of oligonucleotide nuclease degradation (Equation 3.1 and Equation 3.2).

$$\frac{dD}{dt} = -k_{app} D \tag{3.1}$$

$$D = D_0 e^{-k_{app} t} = D_0 e^{-kE_0 t} \tag{3.2}$$

where D is the amount of full-length oligonucleotide at time t, D_0 indicates the amount of full-length oligonucleotide at t = 0, E_0 represents the total protein content, and the coefficient k is the rate constant for the decay of full-length oligonucleotide. We then estimated parameter k of Equation 3.2 using SigmaPlot version 8.02 software (SPSS Inc., Chicago, IL).

RESULTS

Successful antisense or siRNA therapy depends upon the delivery of intact oligonucleotide to the target location inside the cell. Unmodified PO-oligonucleotide is readily degraded by serum or nucleases (Kandimalla et al., 1997). We performed a series of experiments such that we could infer the stability of oligonucleotides with different backbone chemistries and in the presence and absence of dendrimer delivery vehicle.

Oligonucleotide Toxicity Experiments

We used an antisense sequence reported to induce toxicity in model leukemia cell lines (Jiang et al., 1996), delivered the oligonucleotide to cells, and measured viability after 24 and 48 hours. As seen in Figure 3.3, oligonucleotides with the phosphodiester backbone had minimal effect on cell viability; however, PS-oligonucleotides when added to cells resulted in a significant decrease in cell viability (Figure 3.4). These results suggested that naked PO-oligonucleotide was being degraded prior to its arrival at the cell specific location needed to induce toxicity in the leukemia cell line. Therefore, to test if an IL-2R targeted delivery vehicle would increase oligonucleotide delivery and toxicity, cells were treated with oligonucleotides in the presence of an antibody dendrimer conjugate. As shown in Figure 3.5, toxicity after treatment with PS-oligonucleotides in the presence of the delivery vehicle was only marginally greater than toxicity of PS-oligonucleotide alone. In addition, the delivery vehicle alone (at zero concentration of PS-oligonucleotide) was as toxic as the PS-oligonucleotide.

Given the relatively low toxicity of the antisense oligonucleotide in the model leukemia cell line used in these experiments, it was difficult to assess changes in stability of oligonucleotide with backbone modification or in the presence or absence of the delivery vehicle; therefore, a more direct method was chosen to examine oligonucleotide stability.

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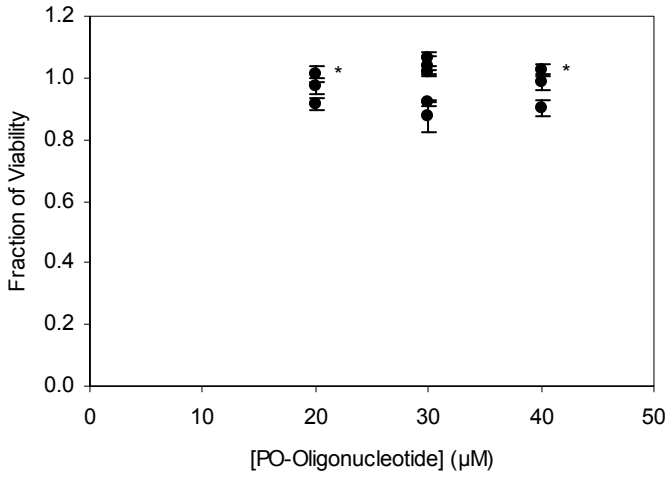

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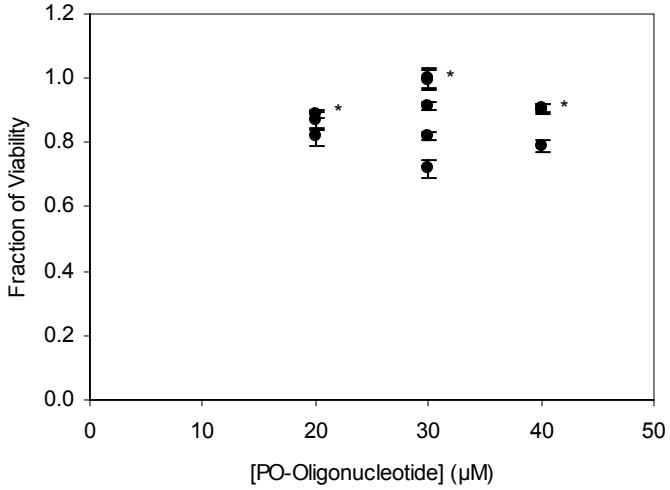
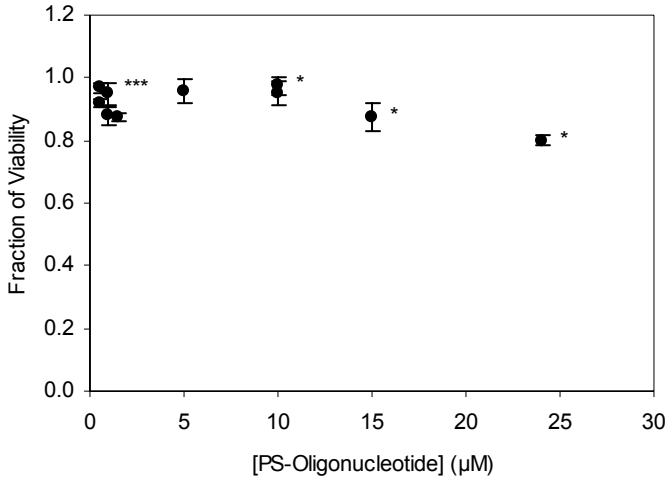


Figure 3.3. PO-oligonucleotide toxicity experiment. The toxicity of PO-oligonucleotide was determined as a function of its concentrations. The data are reported as the fraction of the viability of the cells incubated with the PO-oligonucleotides for (A) 24 and (B) 48 hours relative to the viability of control cells unexposed to the PO-oligonucleotides. The mean $\pm$ standard deviation of 7-8 determinations are presented. For each presented, * indicates measurement is significantly different than for control cells (p<0.05).

A



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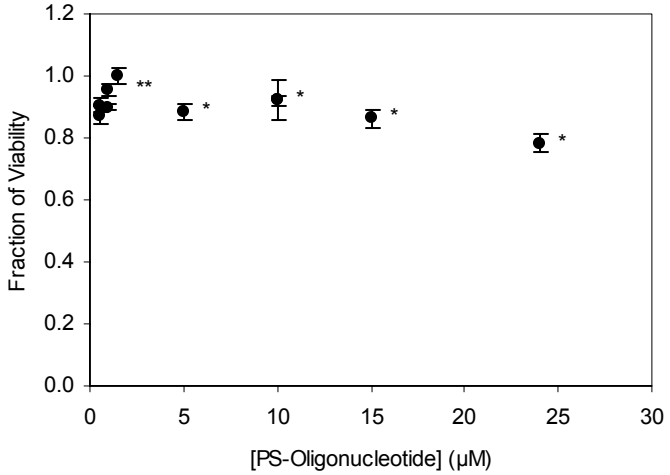
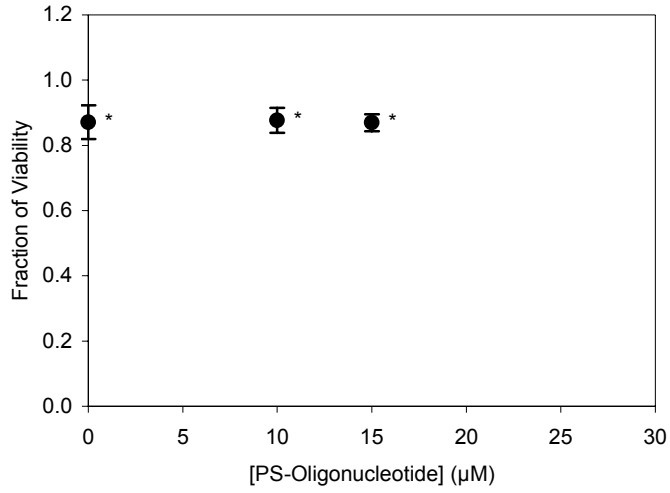


Figure 3.4. PS-oligonucleotide toxicity experiment. The toxicity of PS-oligonucleotide was determined as a function of its concentrations. The data are reported as the fraction of the viability of the cells incubated with the PS-oligonucleotides for (A) 24 and (B) 48 hours relative to the viability of control cells unexposed to the PS-oligonucleotides. The mean $\pm$ standard deviation of 4-6 determinations are presented. For each presented, * indicates measurement is significantly different than for control cells (p<0.05).

A



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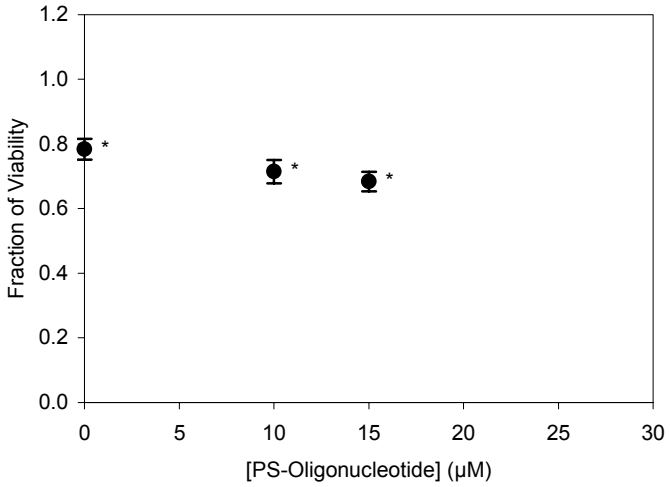


Figure 3.5. Toxicity of PS-oligonucleotide in the presence of antibody dendrimer conjugate. The toxicity of PS-oligonucleotide in the presence of an antibody dendrimer conjugate was determined as a function of oligonucleotide concentrations. The data are reported as the fraction of the viability of the cells incubated with the PS-oligonucleotides in the presence of an antibody dendrimer conjugate for (A) 24 and (B) 48 hours relative to the viability of control cells unexposed to the PS-oligonucleotides with the conjugate. The mean $\pm$ standard deviation of 5-6 determinations are presented. For each presented, * indicates measurement is significantly different than for control cells (p<0.05).

Oligonucleotide Degradation Assay and Parameter Estimation

HPLC analysis was used to identify hydrolysis products formed due to nuclease degradation. Representative chromatograms of PO-oligonucleotide and hydrolysis products in phosphate buffer saline (Figure 3.6A), in fresh medium (Figure 3.7A), in conditioned medium (Figure 3.8A), and in cell lysate (Figure 3.9A) are shown as a function of time exposure to the medium. The relative concentrations of intact oligonucleotides, calculated from peak areas of chromatograms, are shown as a function of incubation time in buffer, fresh medium, conditioned medium, and cell lysate, in Figures 3.6B, 3.7B, 3.8B, and 3.9B, respectively. As seen in Figure 3.6, little or no degradation of oligonucleotide occurred in the phosphate buffer saline solution. These results establish that the oligonucleotide is stable in the absence of nucleases in our study. In Figures 3.7 and 3.8, the rate of degradation of oligonucleotides in fresh and conditioned medium can be seen. Degradation rates were similar in fresh and conditioned medium, and were somewhat modest. In contrast, oligonucleotide degradation in cell lysate was considerable (Figure 3.9). First order rate constants for degradation were estimated in the various medium from data presented in Figures 3.6-3.9. A summary of estimated rate constants, presented in Table 3.1, indicates clear differences in degradation between intra- and extracellular environments.

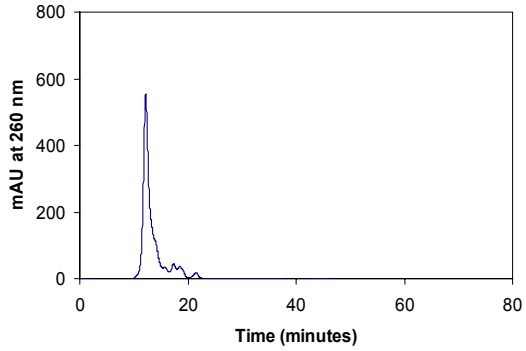
Complex Degradation Assay

The ability of dendrimer to protect the oligonucleotide against nuclease degradation is of great interest. Thus, we were interested in evaluating the ability of the dendrimer delivery vehicle to protect the oligonucleotide from degradation in both intra- and extracellular environments. In order to accomplish this goal, we needed to first develop an HPLC method for the separation of the oligonucleotide and dendrimer prior to measurement of hydrolysis products from oligonucleotide degradation.

A cationic-exchange HPLC method was evaluated for the separation of the oligonucleotide and the dendrimer. It should be noted that, in our study, complexing the oligonucleotide and generation 3.0 dendrimer at a molar ratio equivalent to 1:1 resulted

A

0 hours



4 hours

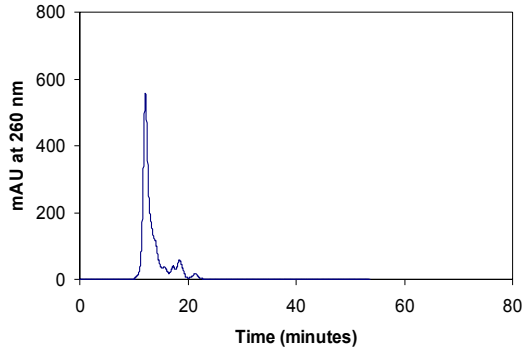
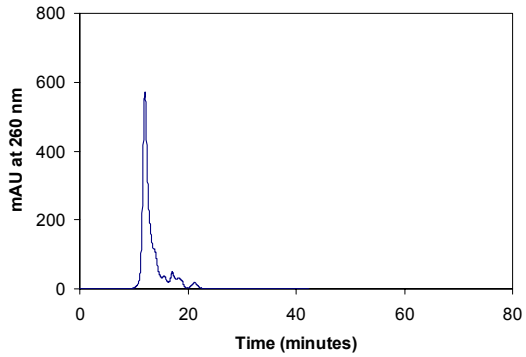
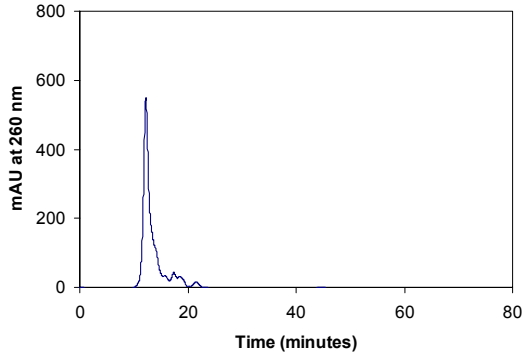


Figure 3.6. Degradation patterns of PO-oligonucleotides in phosphate buffer saline pH 7.2. Degradation was monitored by HPLC. (A) Representative HPLC chromatograms of oligonucleotides as a function of incubation time; (B) Oligonucleotide expressed in units of relative concentration is plotted *versus* incubation time. HPLC was performed with a 20-µL sample loop using 20 mM phosphate buffer containing 250 mM NaCl, pH 7.2 at a flow rate of 0.1 mL/min. The buffer solution was filtered and degassed by vacuum filtering using a 0.2 µm pore size, 47 mm diameter filter composed of nylon 66 (Rainin, Ridgefield, NJ). Sixty µL of samples were injected and the eluate absorbance was monitored at 260 nm.

8 hours



12 hours



24 hours

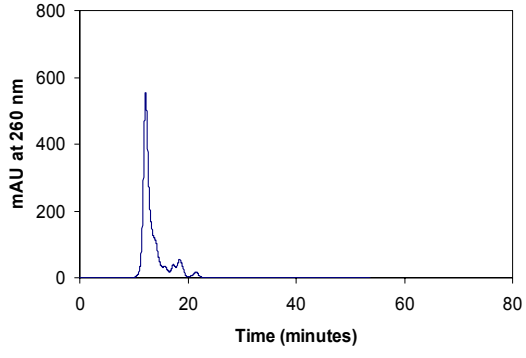


Figure 3.6. (Continued)

B

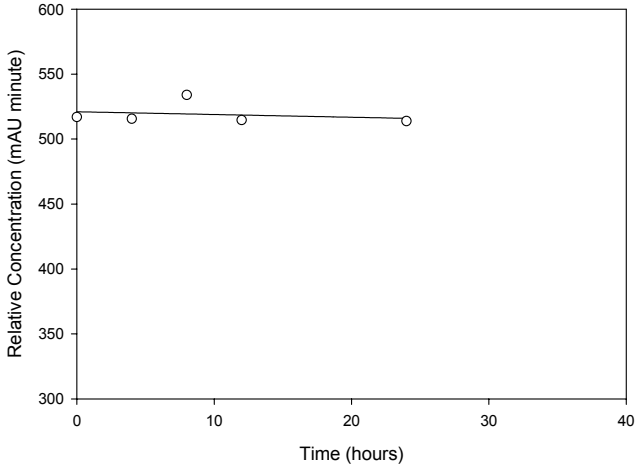
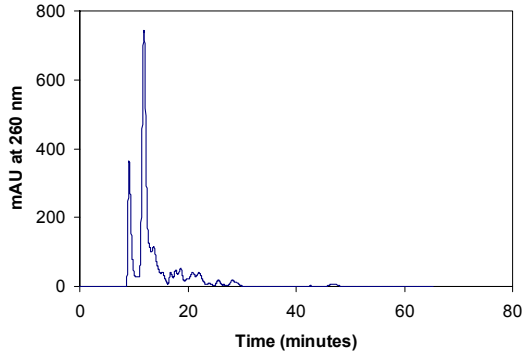


Figure 3.6. (Continued)

A

0 hours



4 hours

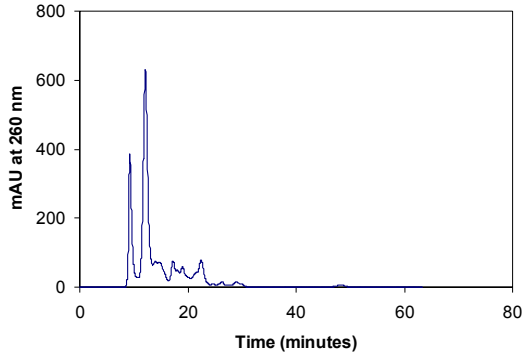
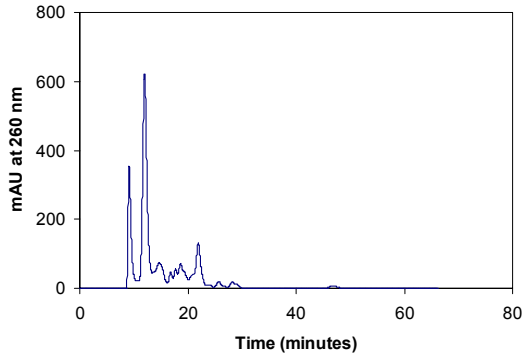
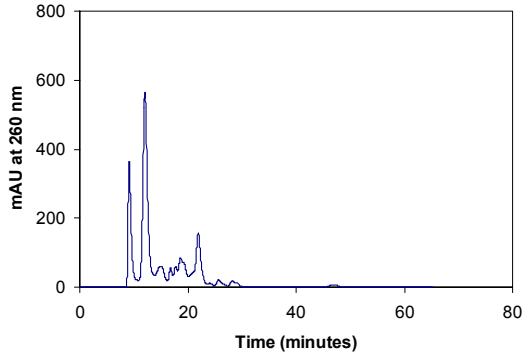


Figure 3.7. Degradation patterns of PO-oligonucleotides in fresh medium. Degradation was monitored by HPLC. (A) Representative HPLC chromatograms of oligonucleotides as a function of incubation time; (B) Oligonucleotide expressed in units of relative concentration is plotted *versus* incubation time. HPLC was performed with a 20-µL sample loop using 20 mM phosphate buffer containing 250 mM NaCl, pH 7.2 at a flow rate of 0.1 mL/min. The buffer solution was filtered and degassed by vacuum filtering using a 0.2 µm pore size, 47 mm diameter filter composed of nylon 66 (Rainin, Ridgefield, NJ). Sixty µL of samples were injected and the eluate absorbance was monitored at 260 nm. PO-oligonucleotides in fresh medium were tested for stability. A BCA assay was performed in order to quantify the protein concentration. The total protein concentration in the fresh medium was 22.14 mg/mL. Measurements were performed in duplicate (sample discrepancies were within 24 mAU·minute units).

12 hours



24 hours



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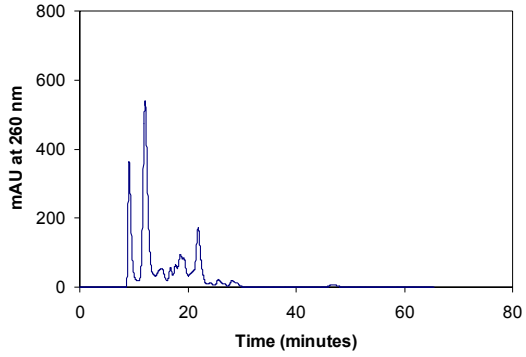

Figure 3.7. (Continued)

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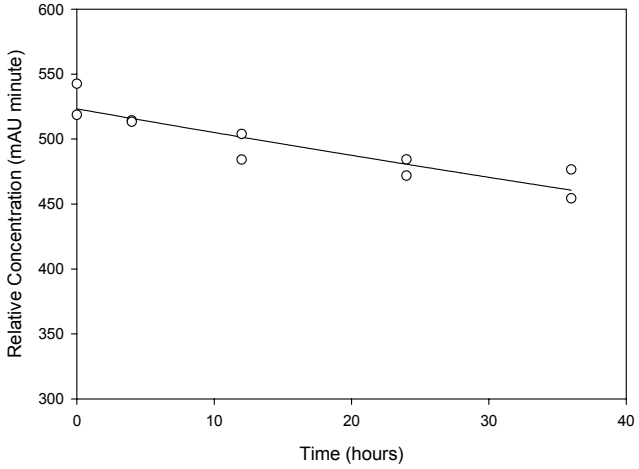

Figure 3.7. (Continued)

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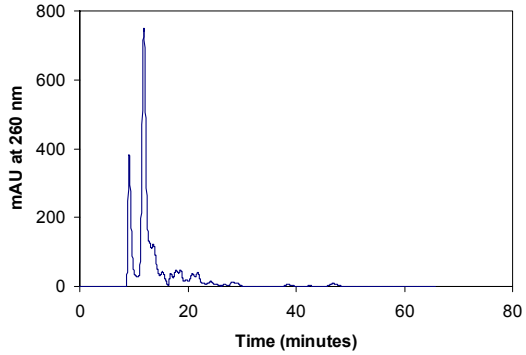

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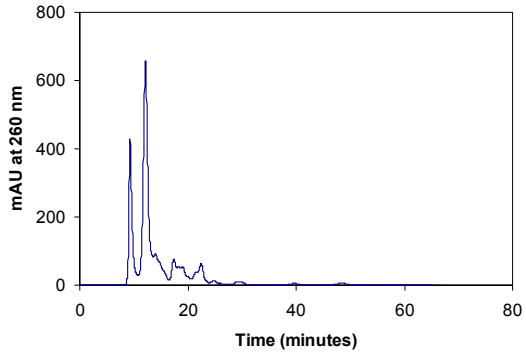
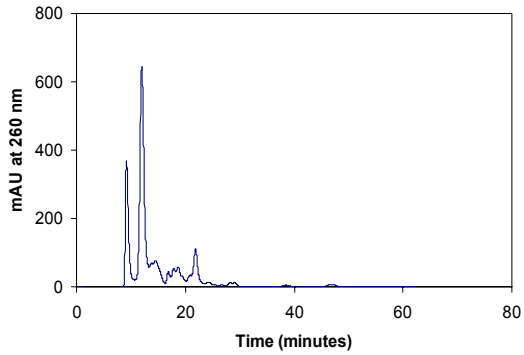


Figure 3.8. Degradation patterns of PO-oligonucleotides in conditioned medium. Degradation was monitored by HPLC. (A) Representative HPLC chromatograms of oligonucleotides as a function of incubation time; (B) Oligonucleotide expressed in units of relative concentration is plotted *versus* incubation time. HPLC was performed with a 20-μL sample loop using 20 mM phosphate buffer containing 250 mM NaCl, pH 7.2 at a flow rate of 0.1 mL/min. The buffer solution was filtered and degassed by vacuum filtering using a 0.2 μm pore size, 47 mm diameter filter composed of nylon 66 (Rainin, Ridgefield, NJ). Sixty μL of samples were injected and the eluate absorbance was monitored at 260 nm. PO-oligonucleotides in conditioned (taken from 3-day cells) medium were tested for stability. A BCA assay was performed in order to quantify the protein concentration. The total protein concentration in the conditioned medium was 18.39 mg/mL. Measurements were performed in duplicate (sample discrepancies were within 24 mAU·minute units).

12 hours



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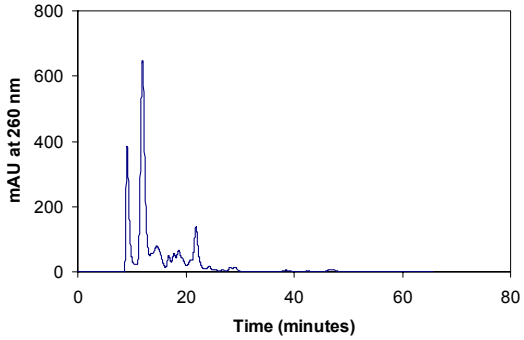

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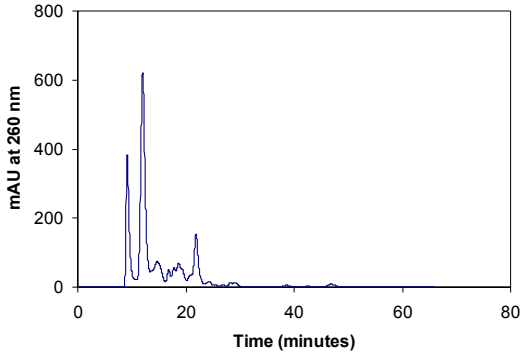

Figure 3.8. (Continued)

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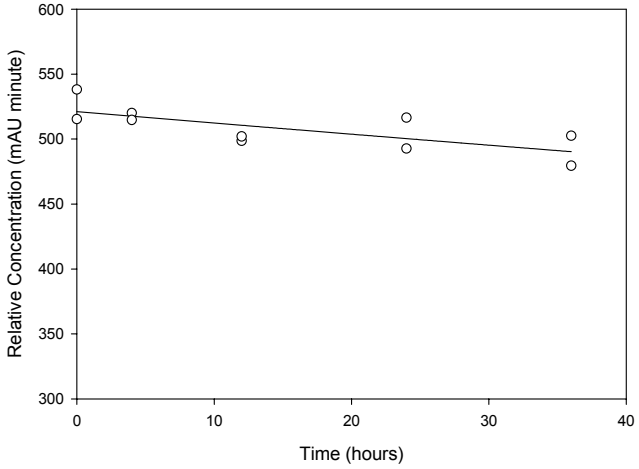

Figure 3.8. (Continued)

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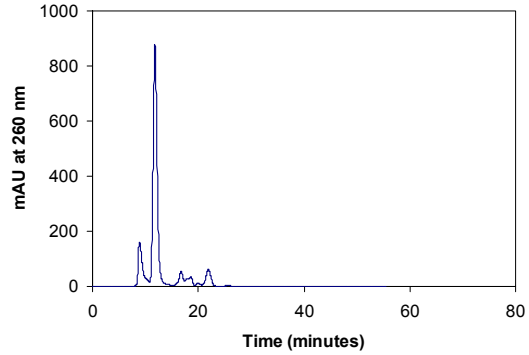

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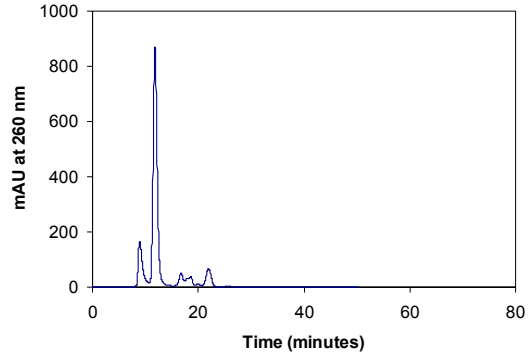

Figure 3.9. Degradation patterns of PO-oligonucleotides in cell lysate. Degradation was monitored by HPLC. (A) Representative HPLC chromatograms of oligonucleotides as a function of incubation time; (B) Oligonucleotide expressed in units of relative concentration is plotted *versus* incubation time. HPLC was performed with a 20-µL sample loop using 20 mM phosphate buffer containing 250 mM NaCl, pH 7.2 at a flow rate of 0.1 mL/min. The buffer solution was filtered and degassed by vacuum filtering using a 0.2 µm pore size, 47 mm diameter filter composed of nylon 66 (Rainin, Ridgefield, NJ). Sixty µL of samples were injected and the eluate absorbance was monitored at 260 nm. PO-oligonucleotides in cell lysate were tested for stability. A BCA assay was performed in order to quantify the protein concentration. The total protein concentration in the cell lysate was 0.3814 mg/mL. Measurements were performed in duplicate (sample discrepancies were within 32 mAU·minute units).

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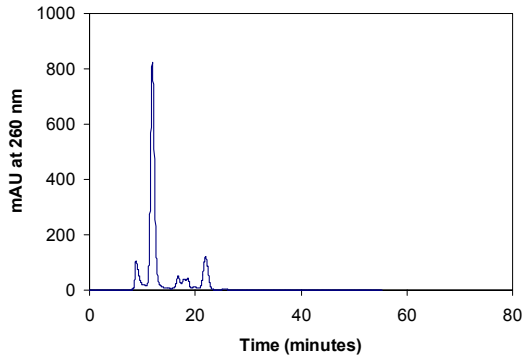

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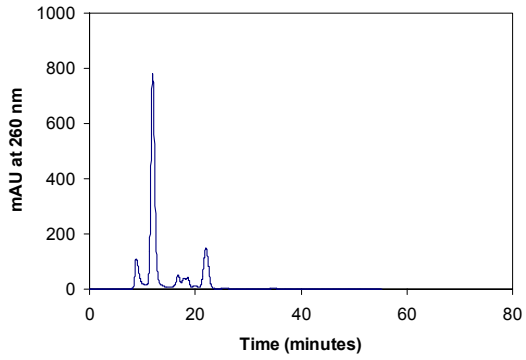

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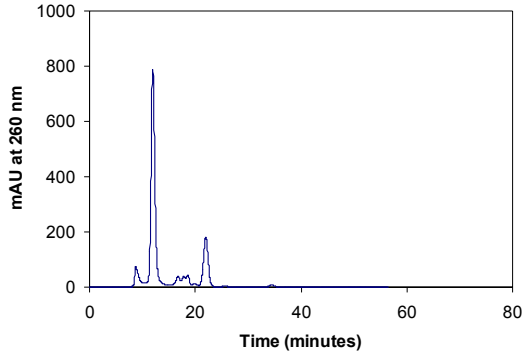

Figure 3.9. (Continued)

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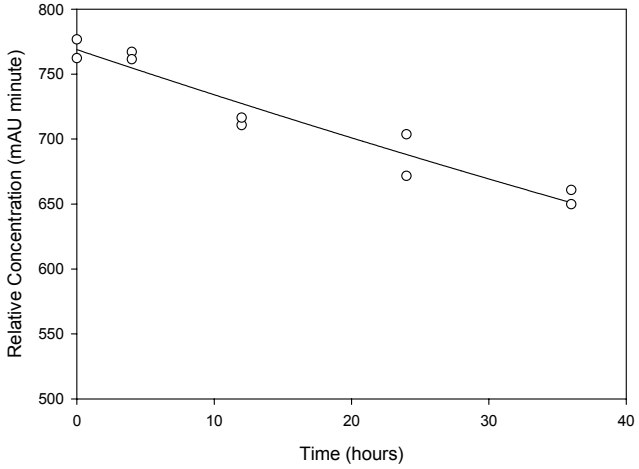

Figure 3.9. (Continued)

Table 3.1. Summary of exponential decay rate constants of PO-oligonucleotide obtained in degradation experiments.

Treatment	**k** $(mg/mL)^{-1}\ hr^{-1}$
Phosphate Buffer Saline pH 7.2	0
Fresh Medium	0.0002 ± 0.00003
Conditioned Medium	0.0001 ± 0.00004
Cell Lysate	0.0121 ± 0.0011

in complex aggregation. Therefore, a complex of 3-fold molar excess dendrimer in TBS at pH 8.0 was used. The electrostatic interaction between oppositely charged species is a process which can be reversed under the increase in ionic strength of the physiological environment, pH change, and dilution. In this work, the separation of oligonucleotide and generation 3.0 dendrimer was achieved on a cationic-exchange guard column using a linear salt gradient. The representative chromatogram in Figure 3.10 shows fully resolved peaks of oligonucleotide and generation 3.0 dendrimer. It is important to note that almost 100% of the oligonucleotide was separated from the complex.

Considerable aggregation of discrete particles was observed in our study. We incubated the dendrimer-oligonucleotide complex in cell lysate in order to examine the ability of the dendrimer to reduce the rate of degradation of the oligonucleotide in the intracellular environment; however, incubating the complexes in lysate produced aggregates that are easily visible to the unaided eye during incubation over several hours. We further examined the aggregation products of the dendrimer complex incubated in the cell lysate such that we could eventually analyze the hydrolysis products of the oligonucleotide formed in this environment. Because this aggregation phenomenon is not as well documented, several attempts had been made in order to dissolve the pellets. The gradually small addition of a base solution to the mixture over the monitoring of change of pH or the addition of known detergents, such as urea and n-octyl-β-D-glucopyronoside, all resulted in oligonucleotide degradation or non-dissolved complexes, respectively. In particular, it is important to note that the amount of intact oligonucleotide was completely degraded at pH 11-13, indicating that the basic pH is responsible for degradation (data not shown). Inability to dissolve the resulting aggregates suggested that the complexes and cell lysate have an inherent tendency toward aggregation under physiological buffer conditions. Therefore, a more complex model of aggregation and/or colloid behavior may be required to describe how cell lysate mediates aggregation of the dendrimer complex.

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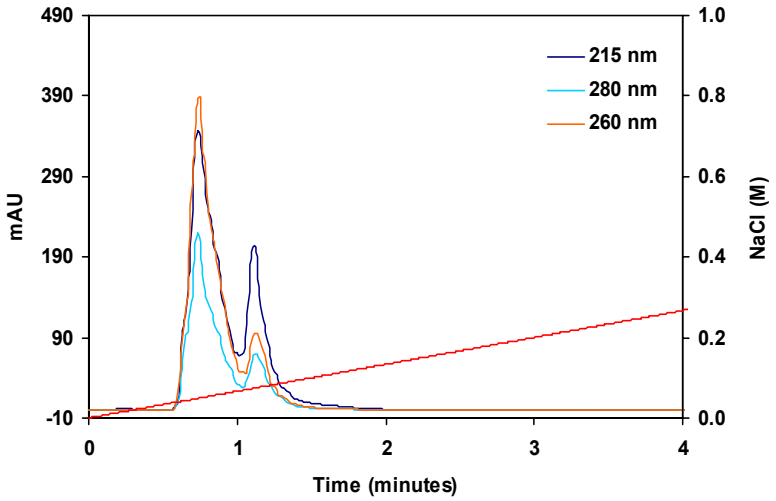


Figure 3.10. Gradient separation of generation 3.0 dendrimer and PO-oligonucleotide by HPLC in cationic-exchange conditions. HPLC was performed with a 250-µL sample loop using a binary buffer system composed of buffer A: 20 mM phosphate buffer, pH 7.0 and buffer B: 20 mM phosphate buffer containing 2 M NaCl, pH 6.8 at a flow rate of 0.5 mL/min. (A) The gradient started at 0-50% B for 0-15 min; it reached 50% B for 15 min. (B) It was then increased to 50-100% B for 0-15 min; it reached 100% B for 15 min. The absorbance at 215, 260 and 280 nm is plotted *versus* time. The buffer solution was filtered and degassed by vacuum filtering using a 0.2 µm pore size, 47 mm diameter filter composed of nylon 66 (Rainin, Ridgefield, NJ). Fifty µL of samples were injected and the eluate absorbance was monitored at 215, 260, and 280 nm. Note that almost 100% of the oligonucleotide, the first peak in (A), was separated from the complex.

B

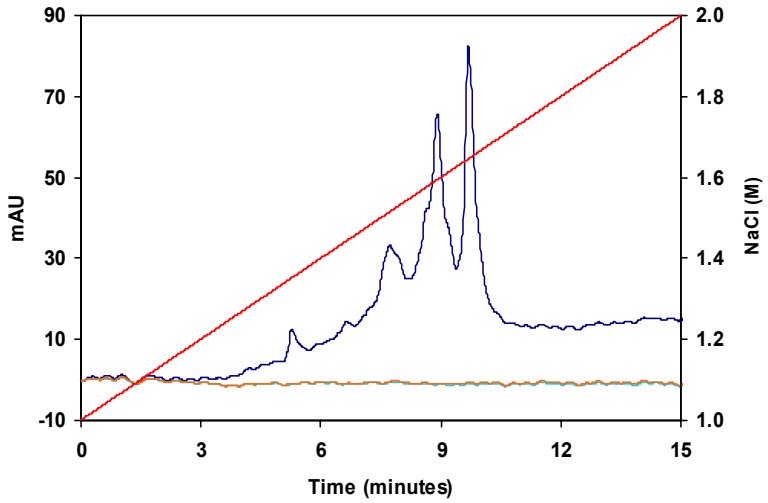


Figure 3.10. (Continued)

DISCUSSION

Recent rapid development in functional genomics has led to a rapid expansion in identifying defective genes that cause a variety of human diseases. Gene therapy and antisense approaches have emerged as novel therapeutics and have provided an effective treatment to inhibit synthesis of those defective gene products as a cure for diseases. Antisense oligonucleotides hybridize with complementary cellular DNA or RNA sequences and inhibit a sequence-specific gene expression either in the nucleus or in the cytoplasm, resulting in repression of protein expression.

Several investigators have proposed a conceptual cellular delivery system in which uptake of oligonucleotides is driven through an energy-dependent mechanism. Endocytosis (receptor-mediated, adsorptive and fluid-phase or pinocytosis) and non-endocytosis associated with specific proteins are believed to be involved in the cell membrane transport and cellular internalization of oligonucleotides (Cotten et al., 1990; Gewirtz et al., 1996; Wu-Pong et al., 1997; Garcia-Chaumont et al., 2000; Lou et al., 2001; and others). Upon internalization, endosomal compartments undergo continuous acidification from the initial cell surface pH (~7) to that found in lysosomes (~4) through the action of ATP-dependent proton pumps acting in conjunction with other ion transporters found in the membrane. Endocytosed oligonucleotides then escape the endosomal and lysosomal compartments and bind to the mRNA targets for transcription (Phillips and Gyurko, 1997; Varga et al., 2000). Upon entering the nucleus, oligonucleotides are rapidly bound to the nuclear matrix in the process of transcription (Shi and Hoekstra, 2004). The progress and ultimate success in any of these steps associated with delivery of oligonucleotide to target site within the cell and reduced expression of the protein of interest is highly dependent upon the stability of the oligonucleotides in both extracellular and intracellular compartments.

In this study, we delivered single-stranded DNA in the form of PO- and PS-antisense oligonucleotides to specific to the translation initiation region of the human B-lymphoblastoid cell line mRNA encoding PDE1B1, which is selectively expressed in leukemic and actively growing lymphocytes (Jiang et al., 1996) without cross-reaction

with other sequences in the human genome. We first proposed to examine stability of oligonucleotides as function of backbone substitution and presence or absence of dendrimer delivery vehicle by examining the toxicity of the oligonucleotide on the model leukemia like cell line. We expected backbone substitution and use of the delivery vehicle to enhance toxicity but enhancing stability and facilitating the internalization of the oligonucleotide. However, unlike the previous study we cited, the selected oligonucleotides with and without an IL-2R targeted delivery vehicle did not significantly alter cell viability. These results have two major implications: (1) there was a problem in one or more of the many steps necessary for effective oligonucleotide delivery, protein expression reduction, and subsequent loss of cell viability that was expected; and (2) specific experiments will be needed to assess the progress of each individual step in oligonucleotide delivery and protein expression reduction.

There are several possible explanations for why the antisense oligonucleotide chosen for these experiments, which was reported by others to lead to a reduction in viability of a human B lymphoblastoid cell line, did not lead to significant reduction in viability of our model leukemia line (Jurkat cells). We might have selected a less efficient sequence for Jurkat cells or that the suppression of gene expression by that sequence is transient. There may be a possibility that the cells used in our study are difficult to transfect. Moreover, when naked oligonucleotides are added to cells, they may not permeate well across the plasma membrane, most likely due to their negative charge against the negatively charged plasma membrane. In addition, the molecular weight of oligonucleotides used in our study far exceeds 1000 Da which could possibly cause a significant delivery problem. Previous studies have demonstrated that PS-oligonucleotides appeared to enter the endosomes, whereas the PO-derivatives showed relatively short half-lives and could undergo efflux from the cell prior to acidification (Tonkinson and Stein, 1994).

While we presented toxicity data for antisense directed against PDE1B1, we have tried other cellular targets (cyclin D1 and cyclin B1) and approaches for delivery, summarized in Table 3.2. The cyclins are necessary for cell proliferation, and their

Table 3.2. Summary of the toxicities of oligonucleotide and siRNA in the presence and absence of the delivery vehicle. The data are reported as the fraction of the viability of the cells incubated with the oligonucleotides and siRNA for 48 and 24 hours, respectively, in the presence and absence of the delivery vehicle relative to the viability of untreated control cells.

mRNA target	**Sequence**	**Concentration**	**Delivery vehicle**	**Fraction of viability**
Cyclin D1	Oligonucleotide	33.3 μM	None	0.75 ± 0.06
		16.7 μM	Dendrimer	0.71 ± 0.25
Cyclin B1	siRNA	75 nM	None	0.93 ± 0.03
		75 nM	Oligofectamine	0.84 ± 0.05

inhibition should lead to either a reduction in proliferation or loss of viability in cells. Under similar concentration ranges (up to 30 μM), no more than 20% reduction in cell proliferation was ever observed. We also compared the use of antisense oligonucleotides and siRNA, which should act on their cellular targets via different mechanisms. We did not have significant success with either approach. We also compared delivery with lipofectamine (a commercially available transfection agent) versus naked siRNA, with limited differences in proliferation; 7% reduction in proliferation with naked siRNA versus 20% reduction in proliferation with lipofectamine. These results highlight the difficulty in use of antisense and siRNA in leukemia treatment. But even more, they highlight the need for independent measurements of the different mechanistic steps associated antisense or siRNA delivery such that effective oligonucleotide therapies can be engineered.

Antisense PO-oligonucleotides are susceptible to degradation via the endocytic route by exo- and endonucleases present in serum and some intracellular compartments. Studies by Hudson et al. (1996) have proven that oligonucleotides exposed to a mixture of rat liver lysosomal enzymes rapidly degraded as assessed via laser densitometry of autoradiography and reverse-phase HPLC. The rate taken for 50% degradation was found to be approximately 30-50 minutes. These results indicate that the lysosomal compartment is a major site of intracellular degradation.

Here, we reported the development of a size exclusion HPLC analytical technique for measurement of the stability of oligonucleotides. To our knowledge, we are the first group to develop the cationic-exchange HPLC method, as an analytical tool, for the separation of oligonucleotide and dendrimer. The use of HPLC is considered desirable for monitoring stability. By using HPLC, we were able to examine the portions of the oligonucleotide based on the difference in size and/or molecular mass that were degraded during incubation and, thus, give more detailed information on reaction products. Previous studies have evaluated the enzymatic degradation patterns of oligonucleotides by capillary gel electrophoresis along with mass spectrometry (MALDI-MS) to get a better insight on the cleavage reactions (Bruin et al., 1995).

Our *in vitro* results suggest that the naked oligonucleotide, not complexed with dendrimer vehicle, is more stable extracellularly than in the intracellular environment (in cell lysate). Indeed, the proteolytic enzymes and nucleases are rich in the lysosomal compartment. Moreover, while not shown, we have revealed that PS-oligonucleotides exhibit more resistance to nucleases than the PO-derivatives.

Ample evidence exists that suggests that DNA masked by the polycations could be protected against nuclease degradation. In one study, complexation of the plasmid DNA to dendrimer at excess positive charges appeared to be resistant to nucleases, presumably by altering the accessibility of the DNA to nucleases. It is interesting to note that the binding of plasmid DNA to dendrimer only altered its secondary and tertiary structure without fragmentation of the DNA (Bielinska et al., 1997). Furthermore, conjugation of the oligonucleotide with an anionic dendrimer improved its stability against serum nucleases, possibly due to steric hindrance of the branched dendrimer (Hussain et al., 2004). Another study has shown that poly(ethylene glycol)-poly-L-lysine-based complexes as assessed via electrophoresis exhibited complete protection of DNA from nucleolytic degradation. Particularly, its increase in protection was found to depend on the molecular weight of the poly-L-lysine used (Dash et al., 1997). De Oliveira et al. (2000) have confirmed that anionic liposomes were able to protect encapsulated oligonucleotides against *in vitro* and *in vivo* plasma nuclease degradation. In addition, Chiou et al. (1994) have indicated that degradation of oligonucleotides bound to the asialoorosomucoid-poly-L-lysine conjugate, as determined via electrophoresis, radioactivity, and autoradiography, was inhibited approximately 3-6 fold in fresh rat serum obtained from whole blood during 5 hours of incubation, compared with the uncomplexed oligonucleotide. Recently, Ferreiro et al. (2003) have also demonstrated that complexation of oligonucleotides to polycationic carriers increased stability against intestinal nucleases in the gastrointestinal tract, possibly due to its forming a steric barrier to enzymes.

Chemical modification of oligonucleotide is considered as an alternative strategy to improve the nuclease resistance. The end-capping and protection of internal

pyrimidine residues have been reported to improve nuclease stability (Peyman et al., 1997). Other investigators have synthesized the oligonucleotide with a self-forming hairpin to protect them from enzymatic degradation (Djavanbakht Samani et al., 2001). Recent work developed by Murata et al. (2003) has revealed that the 3′,5′-modified oligonucleotide-poly(*N*-isopropylacrylamide) conjugate was approximately 54% more resistant to S1 endonuclease than the unmodified oligonucleotide. It is interesting to note that, in this work, PO-oligonucleotide was grafted into the polymer, suggesting that the stability of oligonucleotide was effectively improved by its polymer modification. Moreover, the grafted oligonucleotide was protected, possibly due to less accessibility of nucleases by steric hindrance of the polymer chain.

In conclusion, we were unable to kill leukemic T cells with the oligonucleotides used in our experiments. Surprisingly, with an antibody dendrimer conjugate, toxicity of oligonucleotides was only marginally effective. Thus, it becomes clear to us that the proper selection and/or design of an appropriate antisense sequence of target sites still remains a major challenge in the successful application of antisense technology. Computer-facilitated screening along with gene walking approaches are further required in order to identify accessible sites and to select effective antisense sequences. Therapeutic progress of antisense technology is closely related to the understanding of cellular delivery process. Utilizing a size exclusion HPLC analysis, we were able to examine the portions of the oligonucleotide based on the difference in size and/or molecular mass that were degraded during incubation. This work contributes to our better understanding of the degradation process associated with the intra- and extracellular environment, and further serves as an exploratory guide for rational design of a gene therapy vehicle.

CHAPTER IV
DEVELOPMENT OF PAMAM DENDRIMER AS A POTENTIAL OLIGONUCLEOTIDE DELIVERY SYSTEM

INTRODUCTION

In order to enhance the efficacy and specificity of delivering exogenous materials into targeted cells, a well-defined delivery approach based on the use of potential carriers (vectors or transfection reagents) is needed. Optimization of delivery vectors is one of the most important keys to the success of gene therapeutic applications (Varga et al., 2000; Ferber, 2001). Typically, a delivery vector should provide efficient uptake and expression of the transgene to a selective cell type without stimulating a significant immune or cytotoxic response. Several recently developed nonviral chemical-based carriers can form condensed complexes with DNA allowing for targeted delivery to specific cell types, increased delivery to the cytosol or nucleus, and dissociation in the cytosol or release in the tissue (Niidome and Huang, 2002). Table 4.1 lists the transfection reagents and their effects. Liposomes, mono- or multi-layered phospholipid vesicles, that are either anionic, neutral, fusogenic or cationic, are currently widely used in laboratory settings for transfection of mammalian cells. Liposomes provide several advantages over conventional transfection methods such as calcium phosphate or electroporation, including enhanced delivery efficiency (Sasaki et al., 2001). However, potential toxicity (Pardridge, 2002) and induction of specific immune response (Niidome and Huang, 2002) are still issues that need resolving. In addition, relatively large size, high surface charge and their accumulation in the reticuloendothelial system limit their usefulness for *in vivo* cell transfection (Lou et al., 2001). Cationic polymers have been extensively used for gene transfection of primary cells; however, their ability based upon transfection procedures most likely depends on the presence of effector agents (Cotten et al., 1990; Strauss, 1998). Recent work developed by Lampela et al. (2004) has shown that the combination of cationic lipids and polyethylenimines result in increased transfection efficacy; however, these results are highly dependent upon reagent

Table 4.1. Representative studies of transfection reagents in gene delivery in eukaryotic cells.

Reagent	**Effects**	**Investigators**
DEAE-dextran precipitation	• High cytotoxicity	• Vaheri and Pagano, 1965
Calcium phosphate precipitation	• High cytotoxicity	• Graham and van der Eb, 1973
Electroporation/Permeabilization	• High cell mortality • Relatively enhanced cellular uptake/Nuclear localization	• Spiller et al., 1998
Viral vectors	• High efficiency • Potentially biological hazard (i.e. mutagenesis)	
Liposomes/Cationic lipids	• Improved efficiency • Less cytotoxicity	• Axel et al., 2000 • Kronenwett et al., 1998
Cationic polymers Dendrimers/Activated dendrimers	• Protonability (i.e. buffering capacity) • High efficiency • Minimal cytotoxicity	• Tang et al., 1996 • Godbey et al., 1999a • Reviewed in Dennig and Duncan, 2002

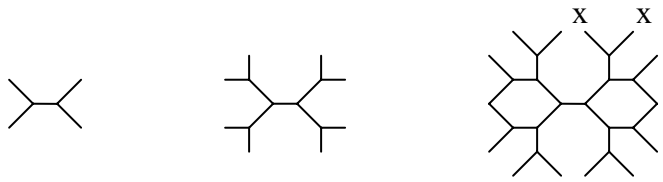
chemistry and cell lines.

The starburst dendrimer (from the Greek: *dendron*, tree; discovered in the late 1970s), a.k.a. polyamidoamine (PAMAM) dendrimer, has received a great deal of attention as a novel nonviral vector with applications in drug delivery and gene therapy (Service, 1995; Cloninger, 2002; and others) (see Figure 4.1). The dendrimers are highly branched three-dimensional macromolecules with defined size, shape, functional terminal surface, and relative physicochemical features comparable to globular proteins (Esfand and Tomalia, 2001). Of particular interest in the functionality and/or molecular interactions, the functional terminal groups of dendrimers have been coupled with a variety of defined chemistries (Fréchet, 1994; Singh, 1998). Dendrimers have been proven to increase gene transfer efficiency at the concentrations required for gene transfer without inducing immunogenicity (Eichman et al., 2000) and cytotoxicity (Haensler and Szoka, 1993; Bielinska et al., 1996). They are shown to facilitate the uptake of oligonucleotides over oligonucleotides unassociated with polymer by 4-5 folds and maintain the functional capacity of associated oligonucleotides (Bielinska et al., 1996). Additionally, stable non-particulate oligonucleotide complexes of generation 5 dendrimer at a high charge ratio in the presence of 30-70% serum have been shown to maintain a relative degree of oligonucleotide activity. The use of a high concentration of serum in culture medium is important in *in vivo* gene therapeutic applications (Yoo et al., 1999).

The importance of the interaction between dendrimer and DNA in successful gene or oligonucleotide delivery has previously been established (Haensler and Szoka, 1993; Bielinska et al., 1996; Dennig, 2003; and others). Dendrimers, which provide the ionic feature of interaction with DNA, induced efficient cellular uptake (Shah et al., 2000). Studies by Kukowska-Latallo et al. (1996) have demonstrated that dendrimers enhanced plasmid DNA transfection and expression in a variety of mammalian cell lines with minimal cytotoxicity.

In this work, we examine properties of a series of PAMAM dendrimers that we believe are associated with success as an oligonucleotide delivery vehicle. The

A



Generation

-0.5	**0.5**	**1.5** (x = COOH)
0.0	**1.0**	**2.0** (x = $CONH(CH_2)_2NH_2$)

B

$NCH_2CH_2N[-(CH_2CH_2CONHCH_2CH_2N)-CH_2CH_2CONHCH_2CH_2NH_2]_4$

generation

$NCH_2CH_2N[-(CH_2CH_2CONHCH_2CH_2N)-CH_2CH_2COOH]_4$

----------|----------|----------

initiator core interior layer terminal unit & group

C

Generation	**Calculated molecular weight (Da)**	**Number of surface groups**	**Number of internal positive groups**	**Diameter (Å) (Tomalia et al., 1990)**
2.0	3,256	16	12	29
2.5	6,267	32	28	--
3.0	6,909	32	28	36
3.5	12,931	64	60	--
4.0	14,215	64	60	45
4.5	26,258	128	124	--

Figure 4.1. Schematic representation of polyamidoamine (PAMAM) dendrimer. (A) Structure and growth. (B) Chemical structure. (C) Calculated molecular weight, number of surface groups, number of internal positive groups, and diameter as a function of generation.

properties we studied include toxicity of the dendrimer delivery vehicle, the binding capacity of the vehicle, and affinity of the vehicle for oligonucleotides. We describe a simple method to determine the binding of oligonucleotides to dendrimers and performed nonlinear regression, as well as parameter estimation to obtain relevant binding properties. We measured the properties of the dendrimer vehicles, toxicity, binding capacity, and binding affinity, as a function of dendrimer generation. These data enable us to begin to evaluate the interplay between different properties of the dendrimer delivery vehicle which will contribute to our ability to design a more effective delivery vehicle for oligonucleotides, siRNA, or genes.

MATERIALS AND METHODS

Materials

Commercially available generation 2.0-4.5 PAMAM dendrimers were purchased from Aldrich (Milwaukee, WI) and used without further purification. Cell culture reagents were purchased from GibcoBRL (Grand Island, NY). 5'-end-fluorescein isothiocyanate (FITC)-labeled oligonucleotide was purchased from Sigma-Genosys (The Woodlands, TX). Aminolink and immobilized diaminodipropylamine (DADPA) gels were purchased from Pierce (Rockford, IL). All other chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, MO).

Cell Culture

Human T-cell leukemia TIB-152 Jurkat cells (ATCC, Rockville, MD) were cultured in a humidified 5% (v/v) CO_2/air environment at 37°C in RPMI supplemented with 10% (v/v) FBS, 10 mM HEPES, 4.5 g/L glucose, 2 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (pH 7.4). For the dendrimer toxicity experiments, cells were plated at densities ranging from 5 to 6 x 10^5 cells/well in 96 well plates.

MTT Reduction Assay

Jurkat cell viability was measured using the MTT reduction assay (Pollack et al., 1995). The dendrimers were incubated with the cells for various lengths of time, after which time MTT was added to the culture medium to yield a final MTT concentration of 0.5 mg/mL. Cells were incubated with the MTT for 4 hours in a CO_2 incubator after which time 100 μL of a 5:2:3 N, N-dimethylformamide (DMF): sodium dodecyl sulfate (SDS): water solution (pH 4.7) was added to dissolve the formed formazan crystals. Then, after 20 hours of incubation in a humidified CO_2 incubator, the MTT absorbance was measured using an Emax Microplate reader at 585 nm (Molecular Devices, Sunnyvale, CA). Viability was reported relative to control cells unexposed to the dendrimers.

FITC-Labeled Oligonucleotide Solution Preparation

Stock solutions of 100 μM were prepared by dissolving the FITC-labeled oligonucleotides in TE (10 mM Tris, 1mM EDTA, pH 8.0). The stock solutions were diluted in phosphate buffer saline (PBS, 13.67 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2) to the concentrations used in the experiment.

Equilibrium Binding Assay

The H_2N-terminated and HOOC-terminated dendrimers were coupled to aminolink and immobilized DADPA gels, respectively, according to the manufacturer's instruction (see Appendix). The FITC-labeled oligonucleotide at various concentrations was added to the dendrimer-linked gels, and the mixtures were then incubated at room temperature for either 30 minutes, 2 hours, or 3 hours. After incubation, the suspensions were centrifuged at 5000 rpm for 2-5 minutes to separate free from bound oligonucleotides. The fluorescence intensity (492 nm excitation and 520 nm emission) of the supernatant was measured to determine the concentration of free oligonucleotide using a Cary Eclipse fluorescence spectrophotometer (Varian Instruments, Walnut

Creek, CA). Analogous experiments were performed using the quenched gels in all other ways.

Estimation of Equilibrium Binding Constant of Oligonucleotides to Dendrimers

Binding of oligonucleotides to dendrimers could be described by a functional form analogous to a Langmuir isotherm. The following system of equations describe the binding in isolation at equilibrium (Equation 4.1) and the total binding as the simple sum of specific and nonspecific binding (Equation 4.2).

$$B_S = \frac{[C_0][F]}{\frac{1}{K} + [F]} \tag{4.1}$$

$$[B_T] = [B_S] + [B_{NS}] \tag{4.2}$$

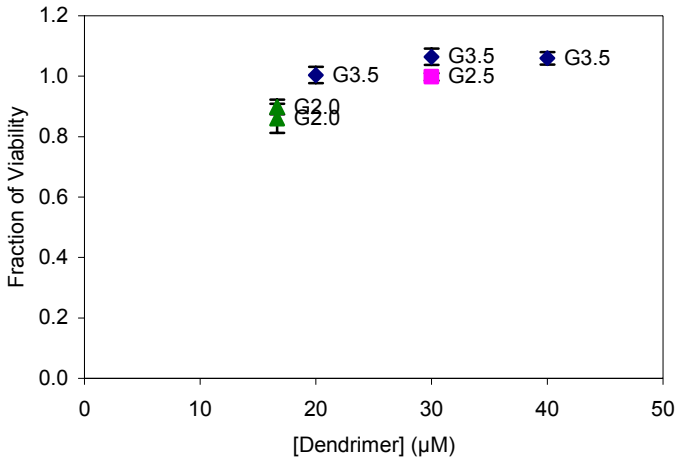
where B_S termed specific binding represents the amount of oligonucleotide bound to the dendrimers, C_0 represents the maximum binding capacity expressed in units of equivalent oligonucleotide concentration, F is the concentration of free oligonucleotide, K is an affinity equilibrium constant, B_T denotes total binding, and B_{NS} is the binding to the quenched gels referred to as nonspecific binding. We then estimated parameters K and C_0 from a non-linear least squares regression of the data corrected for nonspecific binding to the analytic solution of Equation 4.1 using SigmaPlot version 8.02 software (SPSS Inc., Chicago, IL).

RESULTS

Dendrimer Toxicity Experiments

The toxicity of dendrimers was determined as a function of generation at different concentrations of dendrimer. The data were reported as the fraction of the viability of the cells treated with the dendrimers for 24 and 48 hours relative to the viability of control cells unexposed to the dendrimers. As seen in Figures 4.2A and 4.2B, under all conditions, exposure to the H_2N-terminated dendrimers resulted in cell toxicity. While not shown, toxicity of H_2N-terminated dendrimers increased with higher generation number. Conversely, the HOOC-terminated dendrimers did not alter cell

A



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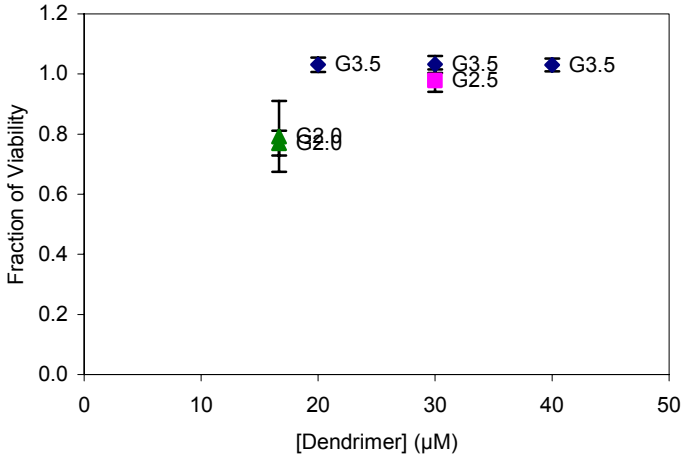


Figure 4.2. Dendrimer toxicity experiment. The toxicity of dendrimers was determined as a function of generation at different concentrations of dendrimer. The data are reported as the fraction of the viability of the cells incubated with the dendrimers for (A) 24 and (B) 48 hours relative to the viability of control cells unexposed to the dendrimers. The mean $\pm$ standard deviation of 8-16 determinations are presented.

viability relative to untreated controls, regardless of generation number. These results indicate that toxicity of dendrimers was dependent upon surface charge.

Equilibrium Binding Assay

In order to estimate the equilibrium constant of the dendrimers for oligonucleotide delivery, we performed an equilibrium binding assay. Preliminary experiments indicated that equilibrium binding was achieved in all cases within 2 hours. For all gels, both specific and nonspecific binding was estimated. For an aminolink gel, the nonspecific binding of oligonucleotide to the gel was negligible, while for an immobilized DADPA gel, isotherms were corrected for nonspecific binding (see Appendix).

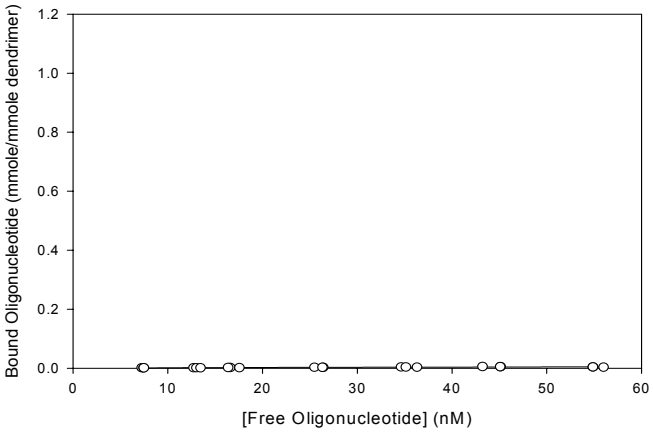
The oligonucleotide binding capacity, or maximum amount of oligonucleotide that the dendrimer vehicle could carry, increased with dendrimer generation for the amine terminated dendrimers; however, binding capacity did not appear to increase with size for the carboxylic acid terminated dendrimers (Figure 4.3 and Table 4.2). For comparable size dendrimers, the amine terminated dendrimers had greater binding capacity than the carboxylic acid terminated dendrimers.

The binding capacity of the same dendrimer was also examined as a function of the ionic strength of the buffers. Binding of oligonucleotides to dendrimers was greater at low salt than at physiological salt concentrations (Figure 4.4).

Estimation of Equilibrium Binding Constant of Oligonucleotides to Dendrimers

Figure 4.3 shows the best fits of our model equations to the experimental data. Using the nonlinear least squares regression of the data corrected for nonspecific binding to Equation 4.2, the values of two parameters, K and $C_{0,}$ were determined. Table 4.2 summarizes the model parameters for the binding of oligonucleotides to dendrimers at equilibrium. While total binding capacities for dendrimers differed depending upon dendrimer size and surface group, equilibrium binding affinity was comparable for all dendrimers tested.

A



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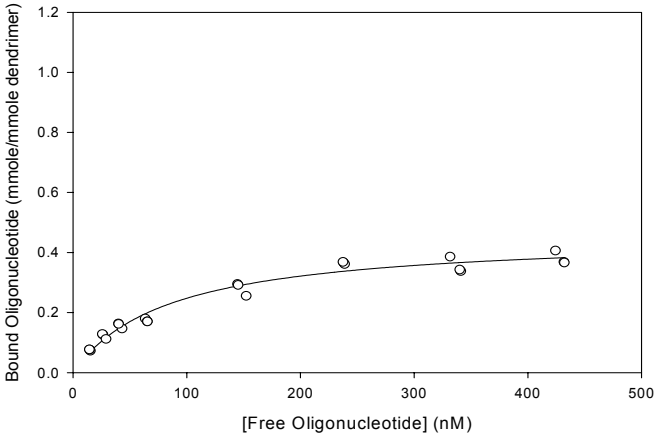
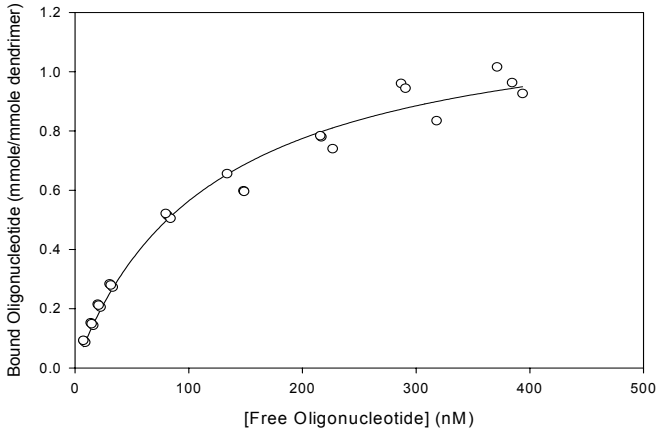


Figure 4.3. Binding of FITC-labeled oligonucleotides to dendrimers. Experiments were conducted at room temperature in phosphate buffer saline (PBS, 13.67 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4) at pH 7.2. Equilibrium binding of the FITC-labeled oligonucleotide to dendrimers is plotted as mmole·(mmole dendrimer)$^{-1}$ *versus* concentration. (A) Generation 2.0. (B) Generation 3.0. (C) Generation 4.0. (D) Generation 3.5. (E) Generation 4.5. The lines represent the best fit of Equation 4.1 to the data.

C



D

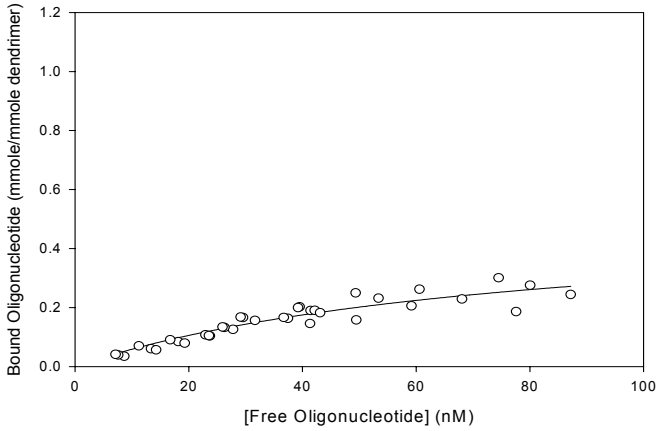


Figure 4.3. (Continued)

E

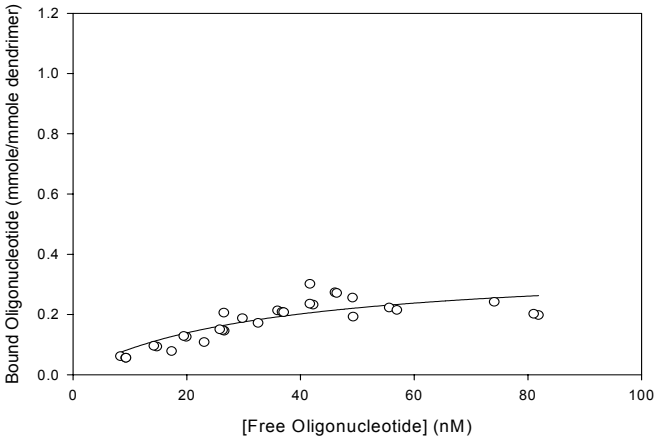
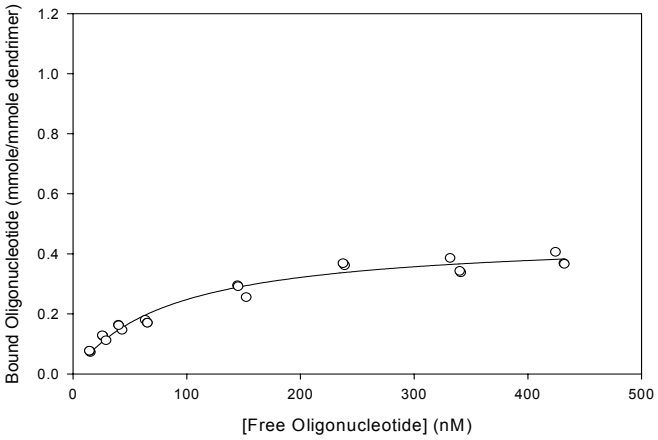


Figure 4.3. (Continued)

Table 4.2. Summary of model parameters obtained in equilibrium binding experiments.

Generation	**K** **M^{-1}**	**C_0** **mmole·(mmole dendrimer)$^{-1}$**
2.0	$2.1 \pm 0.8 \cdot 10^7$	0.009 ± 0.002
3.0	$1.2 \pm 0.1 \cdot 10^7$	0.46 ± 0.02
3.5	$1.3 \pm 0.3 \cdot 10^7$	0.51 ± 0.08
4.0	$8.4 \pm 0.9 \cdot 10^6$	1.24 ± 0.05
4.5	$3.0 \pm 0.9 \cdot 10^7$	0.37 ± 0.05

A



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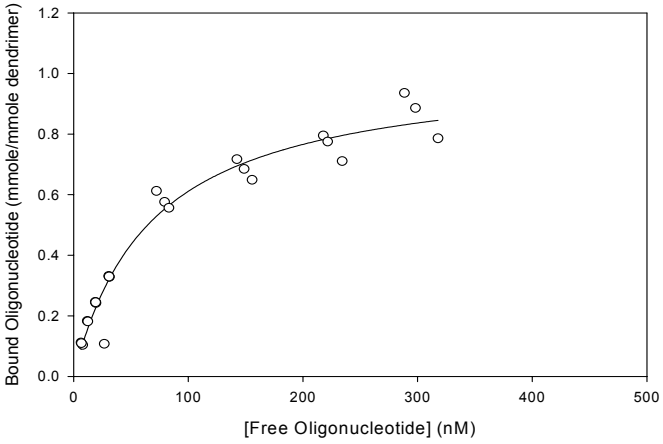


Figure 4.4. Effect of salt on binding of FITC-labeled oligonucleotides to generation 3.0 dendrimer. Experiments were conducted at room temperature in phosphate buffer saline (PBS, 13.67 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4) at pH 7.2 and phosphate buffer (10 mM Na_2HPO_4, 1.76 mM KH_2PO_4) at pH 7.2, respectively. Equilibrium binding of the FITC-labeled oligonucleotide to dendrimers is plotted as mmole·(mmole dendrimer)$^{-1}$ *versus* concentration. (A) Phosphate buffer saline (0.13 M ionic strength). (B) Phosphate buffer (0.01 M ionic strength). The lines represent the best fit of Equation 4.1 to the data.

DISCUSSION

One strategy for developing a gene or oligonucleotide delivery vehicle is to develop a vehicle that changes the physical properties of the oligonucleotide or gene such that its delivery to the target cell (and location of choice within the cell) is facilitated. Numerous barriers must be overcome to achieve biological activity of the delivered oligonucleotide (inhibition of protein expression for antisense or siRNA) into targeted cells. Formation of a complex between the delivered oligonucleotide and a carrier is considered as one of the important steps for nonviral gene delivery. PAMAM dendrimers which possess primary amine or carboxylate groups on the surface and tertiary amines in the interior, as potential carriers, interact electrostatically with the nucleotide phosphates on oligonucleotides at physiological pH to form a compact particle and neutralize its charge.

The biological properties of dendrimers, especially their toxicity and immunogenicity, are considered to be of critical importance for their use in biological applications. Studies by Roberts and coworkers have indicated that *in vitro* toxicity of dendrimers appears to be concentration and generation dependent. In this study, V79 fibroblast cells were exposed to generation 3.0, 5.0 and 7.0 dendrimers at a concentration of 0.1-1000 μM for 4 and 24 hours. Lower generations affected cell growth only at high concentrations, whereas the generation 7.0 dendrimer caused cell death at all the concentrations tested. Moreover, no evidence of immunogenicity of any dendrimer generation tested was observed (Roberts et al., 1996). Malik et al. (2000) examined the structure-biocompatibility relationship, and suggested that PAMAM dendrimers bearing the amine surface above a concentration of 1 mg/mL as assessed via MTT reduction and haemolysis assays were toxic and induced haemolysis after 72 hours and 1 hour exposure, respectively. However, the carboxylate terminated derivatives were less toxic up to a concentration of 2 mg/mL. *In vivo* bio-distribution studies have shown that, owing to inherent toxicity, higher generation cationic dendrimers would unlikely be suitable for parenteral administration. In our toxicity experiment, as seen in Figures 4.2A and 4.2B, we found that exposure to the amine terminated dendrimers resulted in

cell toxicity, in accord with the above findings. We suggest that it is likely due to their highly polycationic nature. The positively charged dendrimers likely interact directly with the negatively charged cell membrane, causing disruption of essential membrane properties associated with cell survival.

Several investigators have previously attempted to characterize physical stability and the particle size of the oligonucleotide complexes (Tang and Szoka, 1997; Ferreiro et al., 2001). In one study, the biophysical properties of the interaction between different cationic polymer structures and DNA were examined using electron microscopy and dynamic light scattering. The apparent diameter and extent of aggregation of the compact structures in solution, referred to as toroidal complexes of 40-60 nm in diameter, strongly depend upon the characteristics of the individual cationic polymer and its charge ratio. More importantly, the apparent binding of cationic polymers to DNA is a linear function of solution ionic strength, which has proven that the interaction between cationic polymers and DNA is predominately electrostatic (Tang and Szoka, 1997). Another study by Ferreiro et al. (2001) has shown that stability of oligonucleotides with poly-L-lysine and protamine salts is pH dependent as determined via the zeta potential analysis. Moreover, an increase in the ionic strength likely promoted dissociation of the complexes. The DNA dissociation characteristics of the polymer complex once present in the cytosol and/or nucleus is essential from the therapeutic point of view (Arigita et al., 1999).

In this study, we examined binding properties of oligonucleotides to a series of dendrimers from generations 2.0-4.5 and estimated the equilibrium binding capacities and association constants for the binding at equilibrium. Our assay technique is accurate and simple and is, therefore, important in studying binding interaction. As seen in Figure 4.3 and Table 4.2, binding capacity of oligonucleotides to dendrimers is a strong function of generation number. Protonation of primary amines and higher order amines at neutral pH could account for the binding of negatively charged oligonucleotides to the highly branched polymers (Tang and Szoka, 1997). For amine terminated dendrimers, the number of positive charges on the dendrimer roughly doubles with every generation.

While binding capacity roughly doubles between generation 3.0 and 4.0 dendrimers, in accord with the increase in charge, there is a much more dramatic increase in binding between generation 2.0 and 3.0. Possibly, generation 3.0 and 4.0 dendrimers have a relatively open and flexible structure compared to generation 2.0, which allows oligonucleotides to enter and reside in the large charged interior, thus facilitating its binding of oligonucleotides. For the carboxylic acid terminated dendrimers, the same increase in binding capacity with size and increased number of internal positive charges was not observed, possibly because the surface charges contribute to binding capacity much more significantly than the internal charges on the polymer.

As shown in Figure 4.4, binding affinity of oligonucleotides to dendrimers is influenced by the salt concentration. Various research groups have previously demonstrated the electrostatic nature of the interaction of the DNA and polycations (Pelta et al., 1996; Bielinska et al., 1999; Plank et al., 1999). Precipitation of DNA and resolubilization of its aggregates induced by subsequent addition of polyamines is a fully reversible process. Interestingly, precipitation appears to be dependent on DNA concentration between 1-50 μg/mL (Pelta et al., 1996). Bielinska et al. (1999) have fractionated aggregated dendrimer-DNA complex and then characterized physiochemical properties involved with the formation of this non-uniform complex. In particular, formation of high-density and less soluble complexes was associated with the complex charge ratio and high DNA concentrations ranging from 0.04 to 1 μg/μL. Electrostatic charge related effects of charged particles appear to be modulated by the generation (i.e. size) of dendrimers. Based on their work, it is likely that dendrimers form neutralized complexes with DNA in a stoichiometry that in primary amines to DNA phosphates is equal to one.

Plank et al. (1999) have synthesized and characterized binding and compacting characteristics of a series of branched cationic peptides that differ in the number and type of cationic amino acids. Peptide affinity for DNA increased with the number of cationic groups in the polypeptide, and a minimal chain length of six to eight cationic amino acids was necessary for the formation of DNA complexes. The peptide

compacted DNA into a microparticulate structure, and its compacting potency has been shown to be structure dependent as determined via an ethidium bromide displacement assay, dynamic light scattering, and electron microscopy. The estimated association constants of branched peptides based on a competitive binding assay were found to be in the range of 10^6 M^{-1} to 10^9 M^{-1}. These equilibrium association constants are of the same order of magnitude that we found for the PAMAM dendrimers tested in this work. We found; however, that equilibrium association constant did not change with dendrimer charge (and that only maximum binding capacity was a function of charge). We speculate, in our work, that the association constant (or binding affinity) is a function of type of polycation (primary or tertiary amine), while binding capacity is a function of total charge of the dendrimer.

In summary, we examined the dendrimer toxicity, binding capacity, and binding affinity with oligonucleotides under physiological and low ionic strength conditions. We presented a simple novel dendrimer-linked gel-based method to obtain equilibrium isotherms and estimated the binding constants for dendrimers. Previous studies have developed the nitrocellulose-filter binding to determine protein-nucleic acid interactions (Wong and Lohman, 1993). Formation of a complex between the delivered gene and a carrier is considered as one of the important steps for nonviral gene delivery. The binding affinity between carrier and gene or oligonucleotide has been implicated in the stability of the complex toward dissociation in an intra- and extracellular environment. These results may help to design better therapeutic vectors for gene or oligonucleotide delivery.

CHAPTER V
KINETIC ANALYSIS OF AN ANTIBODY DENDRIMER CONJUGATE FOR OLIGONUCLEOTIDE DELIVERY

INTRODUCTION

Several investigators have proposed a conceptual cellular delivery system in which uptake of oligonucleotide is driven through an energy-dependent mechanism. Endocytosis (receptor-mediated, adsorptive and fluid-phase or pinocytosis) and non-endocytosis associated with specific proteins are believed to be involved in the cell membrane transport and cellular internalization of oligonucleotides (Cotten et al., 1990; Gewirtz et al., 1996; Wu-Pong et al., 1997; Garcia-Chaumont et al., 2000; Lou et al., 2001; and others). Upon internalization, endosomal compartments undergo continuous acidification from the initial cell surface pH (~7) to that found in lysosomes (~4) through the action of ATP-dependent proton pumps acting in conjunction with other ion transporters found in the membrane. Endocytosed oligonucleotides then escape the endosomal and lysosomal compartments and bind to their mRNA targets for transcription (Phillips and Gyurko, 1997; Varga et al., 2000). Studies by Yakubov et al. (1989) have shown that an endocytosis mechanism is found to be concentration dependent.

A number of investigators have used antibodies to target oligonucleotides to specific cell surface receptors on diseased cells. Once bound to the cell surface receptor, receptor mediated endocytosis leads to internalization of the oligonucleotide in the specific cell population. Suh et al. (2001) have targeted thiolated poly-L-lysine to a PDPH-modified antibody reactive against the human T cell leukemia-specific JL-1 antigen for delivery of oligonucleotides to leukemia cells. Based on their work, 0.54 x 10^4 of JL-1 antigen are found to be present within the cell. They showed that an antibody complex was effectively internalized into cells, possibly via receptor-mediated endocytosis. In other studies, the polyethylenimine (PEI) anti-CD3 antibody conjugate was shown to promote nuclear localization of oligonucleotide in human lymphoma cell

lines through an endocytic pathway (Guillem et al., 2002). Similar strategies have worked in other cancerous cells, as exemplified by work from Patri et al. (2004) that showed that the anti-prostate specific membrane antigen (PSMA) antibody dendrimer conjugates targeting prostate cancer bind to cells expressing PSMA. Other targeting proteins outside of antibodies can also lead to internalization of oligonucleotides. For example, recent work developed by Hussain et al. (2004) has revealed that trypsin-sensitive surface binding proteins may be involved in energy-dependent mechanism of cellular uptake.

Facilitation of endosomal sorting escape before lysosomal degradation has been pharmacologically proposed to improve the efficacy of oligonucleotide delivery (Lou et al., 2001). In a very elegant study, Mikos and coworkers (Godbey et al., 1999b) have revealed details about endocytotic trafficking of the PEI complex using fluorescent labeling and confocal microscopy. The labeled complex initially attached to the cell surface, formed an aggregate (or patch) which was then endocytosed, and entered the nuclei as an associated complex. Upon nuclear localization, the phospholipid-coated complex either interacted with the nuclear membrane or permeabilized and fused with the nuclear envelope, thus leading to the release of a complex into the nucleus. The ability to protonate PEI makes it an effective buffer within endo-lysosomes, and may facilitate its efficient delivery into the nucleus.

Various research groups have hypothesized potential molecular mechanisms of oligonucleotide uptake by cells when receptor mediated endocytosis and/or antibody targeting is not involved, but instead polycations are used to deliver oligonucleotides to cells. A zipper-like association between the positively charged polycation complex and negative charges on the cell surface resulting in adsorptive endocytosis and membrane destabilization has been proposed as a predominant role in the process of internalization. The effectiveness of dendrimer-mediated transfection is altered relative to complex charge ratio, diameter of the dendrimer, and binding of membrane-destabilizing peptides. In addition, transfection efficiency has been shown to be related to the pKa of the amines in the polymer which probably allow the dendrimer to buffer the pH change

in the endosomal compartment (Haensler and Szoka, 1993). Further studies have found that the dendrimer complex enhances oligonucleotide stability under buffer or ionic strength variation due to charge-based association and increased nuclear delivery (Kukowska-Latallo et al., 1996; Delong et al., 1997). In these studies, based on cell type, dendrimer surface charge has proven to be important in the mechanism of transfection.

In the present study, we use an antibody to the IL-2R to target the dendrimer-oligonucleotide complex to a T cell leukemia (Jurkat) cell line. The use of the IL-2R as a selective marker for more conventional chemotherapeutic agents has been addressed many times in the past (Sobolev et al., 2000). Frankel and coworkers (1995) synthesized a genetically altered IL-2-ricin conjugate to target HUT102 human leukemia T cells bearing the high affinity IL-2 receptor; Linares (2000) used IL-2 to deliver photosensitizers to Jurkat (TIB-152) leukemia T cells expressing the IL-2R α chain; and Foss (2001) tested IL-2 genetically fused to the enzymatically active and translocating domains of diphtheria toxin also using high affinity IL-2 receptor bearing cells. In addition, anti-CD25 immunotoxins (Amrolia et al., 2003; Michalek et al., 2003) have been examined to selectively deplete activated T cells associated with graft versus host disease. The IL-2R is a relatively low abundance receptor (less than 2000 copies per cell in many cancers); however, its attractiveness as a target is in its rapid rate of internalization. *In vitro* studies suggest that internalization of the IL-2 receptors occurs continuously (Duprez and Dautry-Varsat, 1986). The initial rate of internalization of ligands associated with its high-affinity sites is on the order of 10^{-1} min^{-1} (Weissman et al., 1986; Lowenthal et al., 1986).

Here, we measured kinetic and thermodynamic parameters associated with binding and internalization of anti-IL-2R antibody-dendrimer-oligonucleotide complexes that were developed for delivery of oligonucleotides to a model T cell leukemia (Jurkat) cell line that constitutively expresses the IL-2 receptor. We experimentally determined the dependence of binding and internalization on dendrimer size and surface charge. The ability to characterize the effects of different delivery vehicle properties on

oligonucleotide delivery may facilitate the design of a more effective treatment strategy for antisense, siRNA, or gene delivery to IL-2R bearing cells.

MATERIALS AND METHODS

Materials

Commercially available generation 2.0-4.0 PAMAM dendrimers were purchased from Aldrich (Milwaukee, WI) and used without further purification. Cell culture reagents were purchased from GibcoBRL (Grand Island, NY). Alexa fluor 532 protein labeling kit was purchased from Molecular Probes (Eugene, OR). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Pierce (Rockford, IL). All other chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, MO).

Cell Culture

Mouse HB-8555 hybridoma cells (ATCC, Rockville, MD) were cultured in a humidified 5% (v/v) CO_2/air environment at 37°C in RPMI supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 10 mM HEPES, 4.5 g/L glucose, 1 mM sodium pyruvate, 0.025 mM 2-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (pH 7.4). Culture supernatant was collected every 4 to 5 days and stored at 4°C for further antibody purification. Human T-cell leukemia TIB-152 Jurkat cells (ATCC, Rockville, MD) were cultured in a humidified 5% (v/v) CO_2/air environment at 37°C in RPMI supplemented with 10% (v/v) FBS, 10 mM HEPES, 4.5 g/L glucose, 2 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (pH 7.4). Cells were plated at densities ranging from 4 to 5 x 10^5 cells/mL in 96 well plates for binding and internalization experiments.

IL-2 Receptor Monoclonal Antibody Purification

Anti IL-2R (CD25) monoclonal antibody was purified from cell culture supernatant by affinity chromatography using a protein A Sepharose 4 Fast Flow column

(Pharmacia, Piscataway, NJ) via standard techniques (Harlow and Lane, 1988; Montage Antibody Purification Kit and Spin Columns with PROSEP-A Media User Guide, Millipore, Bedford, MA). The culture supernatant from HB-8555 cells was filtered through a 0.22-0.45 μm pore size, 47 mm diameter filter (Millipore, Bedford, MA) and diluted 1:1 (v/v) in binding buffer (1.5 M glycine/NaOH, 3 M NaCl, pH 9.0) before loading. The column was washed with 10 bed volumes of binding buffer to remove unbound contaminants. The bound antibody was then eluted with elution buffer (0.1 M sodium citrate, pH 5.5).

The pH of purified antibody was neutralized using 1:20 (v/v) neutralization buffer (1M Tris/HCl, pH 9.0). The antibody solution was concentrated using the Microcon ultra centrifugal filter device with a 30000 molecular weight cut off membrane (Millipore, Bedford, MA). The concentration and purity of antibody were then determined by the absorbance reading at 280 nm (1 OD is approximately equal to 0.8 mg/mL) and SDS PAGE using the pHastgel system (Pharmacia, Piscataway, NJ), respectively. An antibody was stored at -20°C until use.

Alexa Fluor 532 Antibody Labeling

An antibody at a concentration of 0.4 mg/mL in 0.1 M sodium bicarbonate pH 8.3 was added to the alexa fluor 532 (pH insensitive) reactive dye. The mixture was allowed to react for 2 hours at room temperature, followed by purification using a 15000 molecular weight cut off resin column in phosphate buffer saline (PBS, 150 mM NaCl, 10 mM KH_2PO_4/K_2HPO_4, 0.2 mM sodium azide, pH 7.2). A fraction containing antibody was collected and measured with the absorbance readings at 280 nm and 530 nm to determine the degree of labeling. The approximate fluorophore to antibody ratios were calculated based on the extinction coefficient of alexa fluor 532 and on the average molecular mass of the antibody.

Antibody Dendrimer Conjugation

Solutions of dendrimer and EDC were mixed in conjugation buffer (0.1 M MES,

pH 5.0) in a molar ratio of 1:1. The alexa fluor 532-conjugated antibody was added to the complex mixture and rotated at 60 revolutions per minute at room temperature for 3 hours. An antibody dendrimer conjugate was then purified on a PD-10 desalting column (Pharmacia, Piscataway, NJ) in phosphate buffer saline (PBS, 13.67 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2) and the first peak containing antibody was collected. Unreacted reagents were further removed via ultra filtration through a 30000 molecular weight cut off membrane (Millipore, Bedford, MA). The protein content was then measured with the absorbance readings at 280 nm and 530 nm to calculate the degree of conjugation.

Cellular Binding and Internalization Assays

The interaction of antibody dendrimer conjugates with cells was evaluated using flow cytometry of the fluorescent labeled antibody-dendrimer complex. The alexa fluor 532-conjugated antibody was titrated to estimate the optimal concentration for our procedure used to process the cells (Loken et al., 2000). The amount of antibody was determined and held constant at 10.2 nM.

Human T-cell leukemia TIB-152 Jurkat cells at densities ranging from 4 to 5 x 10^5 cells/mL were incubated in 200 μL of RPMI at 4°C and 37°C for the indicated times with an antibody dendrimer conjugate. Each cell sample was then analyzed by flow cytometry to determine the fluorescence level. The data presented here thus show the amount of total cell associated antibody dendrimer complex, both bound and internalized.

Flow cytometry was performed with a FACSArray bioanalyzer (Becton Dickinson, San Jose, CA) equipped with a green (532 nm) laser to excite alexa fluor 532 (530 nm excitation). The emission of alexa fluor 532 (554 nm) was filtered using a yellow 564-606 nm band pass filter. Typically, 5000-10000 events per sample were collected. The mean fluorescence of the whole cell after subtraction of background fluorescence was reported to represent the degree of delivery.

Model and Parameter Estimation

We assumed that our experimental system could be described by the steady state process and characterized by first order rate constants. Figure 5.1 illustrates the schematic description of the endocytic process. The following system of equations described the mass action kinetics of conjugate binding to cell receptor (Equation 5.1) and internalization (Equation 5.2).

$$Ab - D + R_s \underset{k_r}{\overset{k_f}{\rightleftharpoons}} Ab - D - R_s \tag{5.1}$$

$$Ab - D - R_s \xrightarrow{k_i} Ab - D - R_i \tag{5.2}$$

where *Ab-D* represents the concentration of the unbound conjugate, R_s represents the number of cell surface receptors, *Ab-D-*R_s and *Ab-D-*R_i are the concentrations of the bound and internalized conjugates, respectively, k_f is an association rate constant, k_r is a dissociation rate constant, and k_i is an internalization rate constant.

In order to estimate k_i, we used data obtained from experiments performed at 4°C and 37°C, and the fluorescence associated with the cells was compared, correcting for the change in both rate of binding and equilibrium binding at the two temperatures. We then estimated the parameter k_i from a linear least squares regression of the data to the analytic solution of Equation 5.2.

Estimation of Standard Enthalpy Change of Dendrimer Conjugates

The standard enthalpy change of dendrimer conjugates for the reversible reaction, ΔH°, can be described by the Van't Hoff relation (Equation 5.3).

$$\frac{d \ln K}{dT} = \frac{\Delta H^\circ}{RT^2} \tag{5.3}$$

Equation 5.3 can be integrated on the assumption that ΔH° is constant over the temperature interval. Thus, the result is:

$$\ln \frac{K_1}{K_2} = \frac{\Delta H^\circ}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \tag{5.4}$$

where K is the equilibrium constant, R is the universal gas constant (= 1.987 cal/K

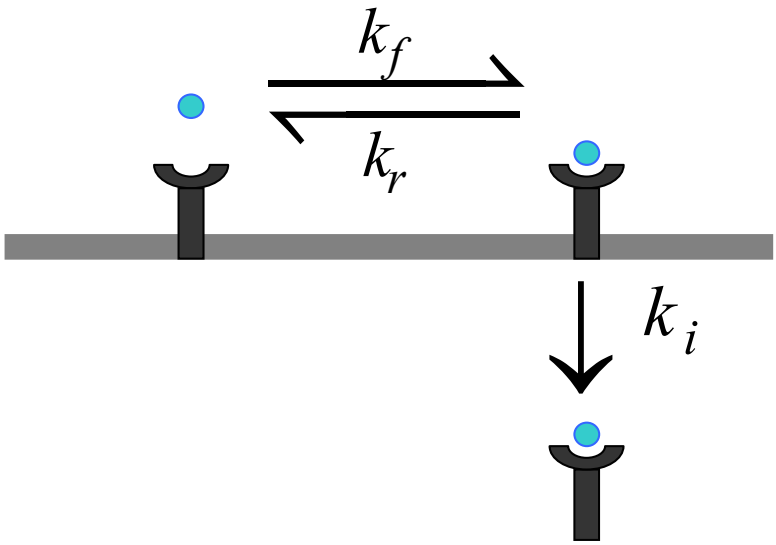


Figure 5.1. Schematic description of the endocytic process. The ligand molecules (circles) bind to the target receptors (block arcs). Receptors and their ligands are internalized into the cell (shaded area represents the plasma membrane).

mole), and T is the absolute temperature in Kelvin. Note that $\Delta H^{\circ} < 0$, K decreases with increasing T, for an exothermic and conversely for an endothermic reaction.
The equilibrium constant can be expressed as:

$$K = \frac{[Ab - D - R_s]}{[Ab - D][R_s]} \simeq \frac{\left(\frac{Average\ of\ Mean\ Fluorescence_{corrected}}{Fluorophore\ to\ Antibody\ Ratio} \right)}{[Ab - D][R_s]} \qquad (5.5)$$

where the concentration of the bound conjugate in Equation 5.5 is defined by the ratio of average of the mean fluorescence after subtraction of background fluorescence to the approximate moles of fluorophore per mole of antibody. The number of cell surface receptors is assumed to be constant. The concentration of the unbound conjugate is constant throughout the experiment. This assumption was based on our procedure that conjugate was used in excess concentrations.

From binding and internalization data at 4°C and 37°C, we estimated apparent equilibrium binding affinities for each of the antibody dendrimer conjugates at the two temperatures (Equation 5.5). From the binding constants we then estimated the standard enthalpy change with binding of the data to the analytic solution of Equation 5.4.

RESULTS

Conjugate Characterization

Several different antibody dendrimer conjugates were prepared using three different H_2N-terminated dendrimers. After preparation and purification, the amount of bound dendrimer to antibody of each conjugate was characterized. The degree of conjugation was determined by the absorbance readings at 280 nm and 530 nm. On the basis of this measurement, it was calculated that the molar ratio of greater than 1000 dendrimer to antibody for conjugates after purification. We believe that the high conjugation ratio reported here is a result of our failure to completely remove unconjugated dendrimer from the conjugated antibody.

Cellular Binding and Internalization

In order to estimate the binding and internalization rate constants of the antibody

dendrimer conjugate used for oligonucleotide delivery to a T cell leukemia line (Jurkat cells), we conducted a fluorescence-based flow cytometry assay. The binding and internalization of antibody dendrimer conjugates prepared with different dendrimer size generations was monitored as a function of time (Figure 5.2 and Figure 5.3). The data were reported as the amount of conjugates bound or internalized per cell. The quantity of conjugate associated with cell was estimated based on fluorescence measurements and calibration beads specific for the fluorophores used in these experiments. In the binding experiments shown in Figure 5.2, in the absence of oligonucleotide, significantly more conjugate prepared with generation 2.0 dendrimer binds to cells than generation 3.0 or 4.0 dendrimer. However, the rate of binding of generation 2.0 dendrimer is slower than the rate of binding of the other conjugates. The data shown in Figure 5.2 was collected at 4°C, a temperature at which receptor mediated endocytosis does not occur.

To determine conjugate internalization, experiments were performed at 4°C and 37°C, and the fluorescence associated with the cells was compared, correcting for the change in both rate of binding and equilibrium binding at the two temperatures. We assumed binding was rapid at 37°C and that internalization was first order. As can be seen in Figure 5.3, internalization for all dendrimer conjugates is low; however the rate of internalization is highest for generation 2.0, then generation 3.0 dendrimer conjugates relative to generation 4.0 dendrimer conjugates, 0.05, 0.04 and 0.02 hr^{-1} respectively, or approximately 70, 20 and 10 conjugates per hour, respectively, for the three conjugates.

Estimation of Standard Enthalpy Change

From binding and internalization data at 4°C and 37°C, we estimated apparent equilibrium binding affinities for each of the antibody dendrimer conjugates at the two temperatures. From the binding constants we estimated the standard enthalpy change with binding as a function of antibody dendrimer conjugates. As shown in Figure 5.4, all conjugates have lower change in enthalpy upon binding to the receptor than the unmodified antibody; however, the generation 4.0 conjugates show the greatest change in enthalpy from the unmodified antibody, suggesting the greatest loss in specificity of

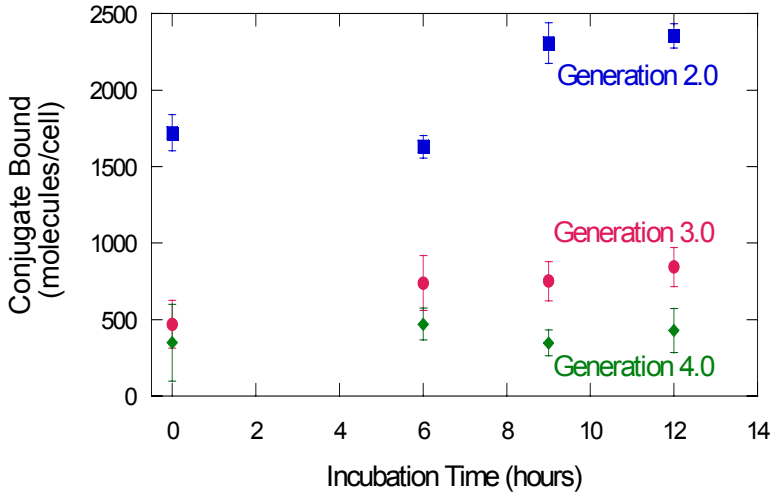
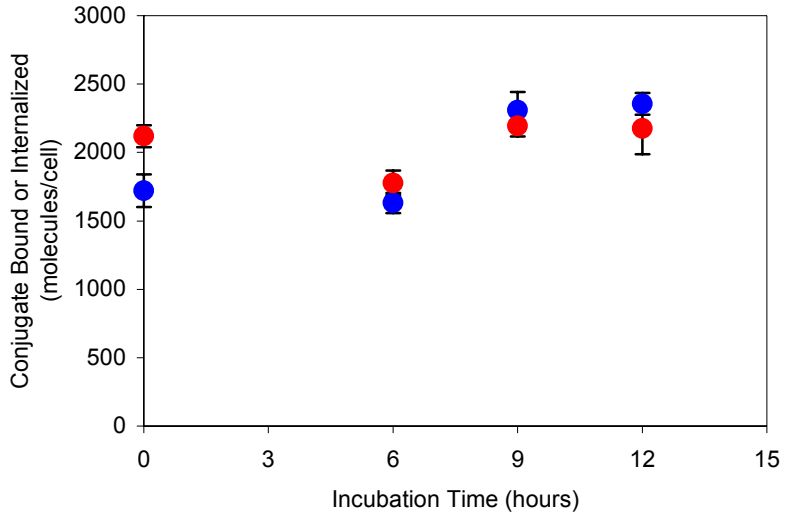


Figure 5.2. Time course of conjugate binding to cells as a function of antibody dendrimer conjugates. Cells were treated with the antibody dendrimer conjugate at 4°C for varying periods of time. The mean ± standard error from 3 to 7 replicates are shown. The amount of conjugates bound per cell is plotted for antibody dendrimer conjugate for dendrimers of generation 2.0 (-■-), 3.0 (-●-), and 4.0 (-♦-).

A



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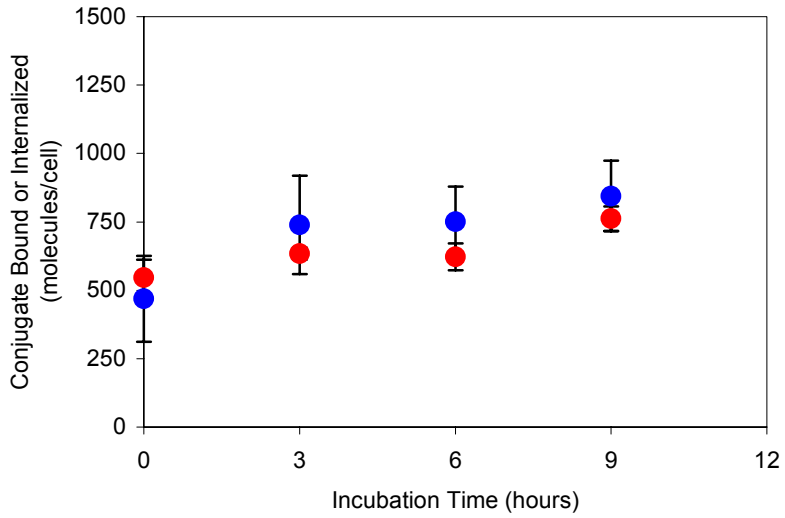


Figure 5.3. Time course of conjugate binding and internalization to cells as a function of antibody dendrimer conjugates. Cells were treated with the antibody dendrimer conjugate at 4°C and 37°C for varying periods of time. The mean ± standard error from 3 to 7 replicates are shown. The amount of conjugates bound (-●-) or internalized (-●-) per cell is plotted for antibody dendrimer conjugate with dendrimers of (A) generation 2.0, (B) generation 3.0, and (C) generation 4.0.

C

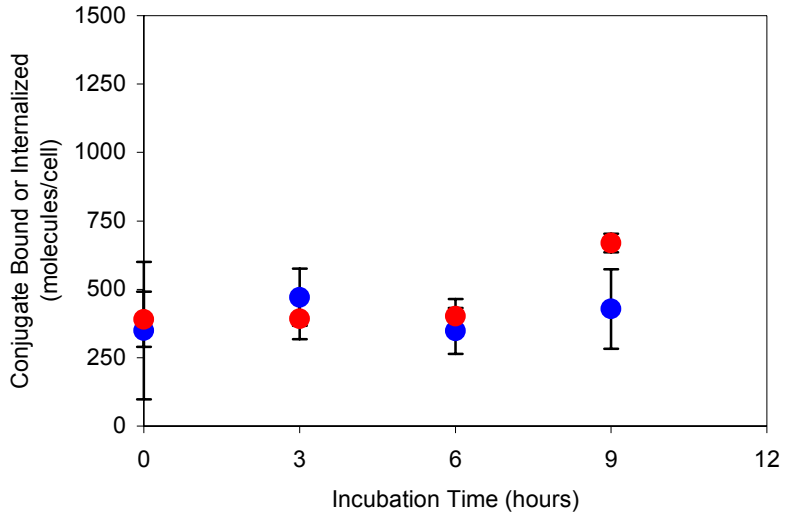


Figure 5.3. (Continued)

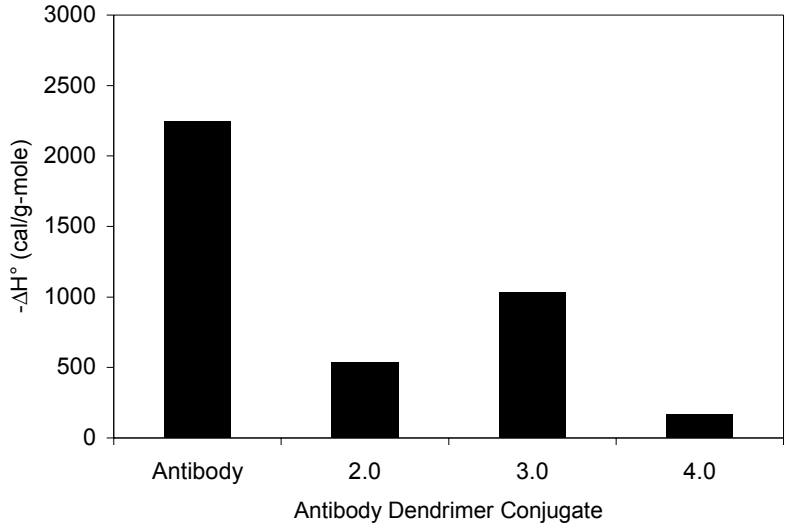


Figure 5.4. Standard enthalpy change as a function of antibody dendrimer conjugates. The standard enthalpy change for the temperature dependence of the equilibrium constant for the antibody dendrimer conjugate for dendrimers of generation 2.0, 3.0 and 4.0 are shown, and are compared with unmodified antibody.

binding of the three types of conjugates prepared.

DISCUSSION

Normal resting T cells do not express the IL-2R α, whereas T cells found in certain lymphoid malignancies and some autoimmune diseases over-express this receptor. This provides the basis to targeted T cell therapies specifically. In this work we examined the kinetic parameters that control effective uptake and intracellular trafficking of an antibody dendrimer conjugate to the IL-2R bearing T cells as part of the proposed therapy. This targeted delivery vehicle could be used to deliver genes, antisense DNA or small interfering RNA to specific diseased cells.

While there have been numerous studies of antibody mediated targeting and delivery of more traditional chemotherapeutics (Blakey et al., 1988), factors that govern effective internalization and delivery of non-charged or protein therapeutics may not apply to highly charged carriers for oligonucleotides. For traditional immunotoxin delivery, internalization and intracellular degradation of the immunotoxin govern (and severely limit) the effectiveness of therapy (Press et al., 1988; Braslawsky et al., 1991; May et al., 1991; Yazdi et al., 1995). However, for delivery of antisense oligonucleotides using cationic polymers or peptides, the high charge of the carriers may facilitate internalization and escape of the oligonucleotides from endosomes.

We examined the binding affinity, enthalpy of binding, and rate of internalization of a number of antibody dendrimer conjugates. In all cases, enthalpy of binding was significantly less than unmodified antibody, probably due to electrostatic interactions associated with the dendrimer. Generation 4.0 dendrimer construct behaved least like the unmodified antibody, suggesting that the greater surface charges associated with the molecule were associated with the change in properties relative to the unmodified antibody. Steric factors may have also played a role in the change in properties associated with dendrimer conjugation. It might be possible that some free dendrimer is absorbed in the conjugate, and that not all dendrimer is covalently linked to the conjugate. The reduction in enthalpy of binding of antibody dendrimer conjugate could

lead to loss of specificity of the antibody for a specific cell surface receptor.

The rate of internalization of dendrimer conjugates was estimated. In all cases, internalization was significantly slower, by more than a factor of 100, than that expected for IL-2 bond to its receptor (Weissman et al., 1986; Lowenthal et al., 1986). The larger size dendrimer-antibody conjugates (generation 4.0) were internalized more slowly than the smaller dendrimer-conjugates (generation 2.0). The reduced rate of internalization may be associated with either charge or molecular size. Numerous lines of evidence have shown that cationic dendrimers interact electrostatically with oligonucleotide forming complexes that are efficiently internalized by endocytosis. The size, structure and overall charge of complexes are critical for efficient delivery from the therapeutic point of view (Maksimenko et al., 2003). Studies by Tomlinson and Rolland (1996) confirm this hypothesis. The transfection efficiency of the dendrimer complex significantly increased as a function of polymer to DNA charge ratio as assessed via β-galactosidase activity. Additionally, Kronenwett and coworkers (1998) showed the enhanced cationic lipid-mediated oligonucleotide uptake in primary hematopoietic cells, which appeared to be cell type dependent with the smallest uptake in $CD7^+$ T cells.

Several investigators have previously elucidated the structure/function relationships of the delivery vectors for gene transfer (Tang et al., 1996; Akinc et al., 2003; Anderson et al., 2004; and others). In one study, the fractured (i.e. partially degraded) PAMAM dendrimer obtained from random solvolysis aided by heat leading to hydrolytic cleavage of some peptide bonds increased transfection efficiency, mainly due to its high degree of flexibility. Indeed, this highly flexible structure acting as a proton sponge, which becomes protonated, swelled and eventually bursted upon uptake in the endosome, would cause endosomal rupture and release of free DNA or the complex into the cytoplasm. Moreover, the protonation of dendrimer would buffer the acidic environment of the endosome, which may inhibit pH-dependent endosomal nucleases (Tang et al., 1996). Audouy and Hoekstra (2001) reviewed that the size of cationic lipid complexes may play an important role in transfection mechanism, likely due to the restricted diameters of endocytic vehicles (~100 nm) and time-dependent clustering of

complexes prior to internalization. In addition, Langer and his teams suggested that small particle sizes and positive surface charges of newly synthesized degradable poly (β-amino esters) led to higher levels of cellular uptake (Akinc et al., 2003). Furthermore, they demonstrated both *in vitro* and *in vivo* use of C32 polymer-based vector, synthesized at 1.2:1 amine per acrylate ratio, to selectively and effectively deliver a DNA construct encoding the A chain of diphtheria toxin to human prostate cancer cells without its associated cytotoxicity. The amino termination has been shown to improve transfection, in this case, suggesting that the extra positive charge may assist DNA complexation or some step in the transfection process (Anderson et al., 2004).

Since efficient delivery of the biotherapeutic agent appears to be linked to endosomal release and sorting, engineering a vector to overcome this limiting barrier can facilitate its escape. Lackey et al. (2002) investigated the use of pH-sensitive poly(propylacrylic acid) as an endosomal-releasing agent to enhance the cytoplasmic translocation of anti-CD3 antibody conjugates across the endosomal membrane in T lymphoma cells.

The overall positive charge of an antibody dendrimer conjugate has been shown to mediate electrostatic interactions with the negatively charged glycosides present on the cell surface. The positively charged complex may increase binding of the negative charge of the cell surface nonspecifically, thereby reducing the efficiency and specificity of a targeted complex. In this regard, it is important to note that the dendrimer may be internalized via fusion or membrane destabilization upon its interaction with the cell surface. Recently, Hong et al. (2004) proposed that the formation of holes, of approximately 15-40 nm, (i.e. dendroporation) generated in the cell membrane by amine terminated dendrimers is responsible for nonselective internalization. Others showed that, using an endothelial cell line as a model, membrane cholesterol and raft (i.e. probably the annular anionic phospholipids surrounding raft) integrity are relevant for the cellular uptake and endocytosis of dendrimer complexes. It should be noted that no nuclear localization of complexes was observed in this study (Manunta et al., 2004). Lauffenburger and coworkers employed biotin-epidermal growth factor (EGF) as the

ligand to deliver plasmid DNA encoding the green fluorescent protein to mouse fibroblasts bearing the human EGF receptor, and revealed that receptor binding of the conjugate, referred to as a rate-limiting step for gene transfer, has been shown to be dependent on the ligand cross-linker spacer length used to prepare the conjugate and link the targeting molecule onto the polylysine conjugate. Additionally, the conjugate charge ratio and valency determine the receptor-specific delivery process (Schaffer and Lauffenburger, 1998). They further provided evidence that dissociation of the polylysine conjugate from the plasmid DNA within the nucleus, as detected via fluorescence microscopy and an *in vitro* transcription assay, has been directly linked to structure and polymer length. These results suggest that vector unpackaging can limit the efficiency of gene delivery and expression (Schaffer et al., 2000). In our work, we also demonstrated that TIB-152 Jurkat cells require the dendrimer conjugate of a certain size, which has less tendency to aggregate, for optimal gene delivery.

In conclusion, we were able to determine the kinetic parameters and to provide information on the binding and internalization of a number of antibody dendrimer conjugates to the IL-2R bearing T cells. We have shown that the dendrimer conjugate of an optimal size, as an endocytosed material, is required for receptor-mediated oligonucleotide delivery. An essential prerequisite for achieving improved oligonucleotide delivery is to understand the whole delivery process. As the delivery mechanisms and kinetic parameters are determined experimentally, it will aid characterization and design of receptor-mediated antisense delivery vehicles. This work contributes to our ability to provide a detailed fundamental understanding of dendrimer conjugate assembly specifically targeting T cells expressing the IL-2R and the associated mechanism of oligonucleotide delivery. This work may aid in our ability to design better vehicles for use in delivery of the oligonucleotides for a variety of therapeutic applications.

CHAPTER VI
MODELING TARGETED GENE THERAPY

INTRODUCTION

The ligand-induced internalization of cell surface IL-2/IL-2R complexes occurs via an endocytic mechanism (Yu and Malek, 2001). Mathematical modeling to examining receptor-mediated internalization has been widely investigated (Gex-Fabry and Delisi, 1984a; Gex-Fabry and Delisi, 1984b; Myers et al., 1987; Chignola et al., 1994; and others). Significant work has been done by Lauffenburger and his teams on modeling ligand/receptor trafficking systems (Lauffenburger and Linderman, 1993; Forsten and Lauffenburger, 1994; Fallon and Lauffenburger, 2000). Here, we have modified existing models to develop a kinetic model of oligonucleotide delivery. The capacity of the delivery vehicle to carry oligonucleotide and affinity of the oligonucleotide to the vehicle were considered in our model. The affinity of the antibody to its receptors is a crucial parameter in the delivery of the oligonucleotide to the target cells. The affinity of the antibody-receptor reaction is dependent on both the association and dissociation rate constants of the antibody for the receptor, parameters considered in this work. The rate of internalization of conjugates, as a rate limiting reaction, plays an important role in our method. Finally, the degradation of the oligonucleotide intracellularly was considered in the model described.

In the model developed, equivalent parameters that could be obtained from experimental data were used to describe the IL-2R targeted therapy. The model can be used to understand better the parameters which govern efficacy and selectivity of a given therapy and to aid in the optimization of therapeutic parameters associated with targeted gene therapy.

MODEL DEVELOPMENT

We propose a one cell model of a T cell that incorporates IL-2R trafficking based on models by Lauffenburger and coworkers (Lauffenburger and Linderman, 1993;

Forsten and Lauffenburger, 1994; Fallon and Lauffenburger, 2000) and by those of Linares (2000). The model uses mass action kinetics to describe the rate expressions of oligonucleotide-antibody dendrimer conjugate binding, binding and internalization of oligonucleotide-antibody dendrimer conjugate to the cell receptor, oligonucleotide release in the endosome, and degradation of the oligonucleotide.

In our model, oligonucleotide (*O*) is subject to degradation in an extracellular environment, O_{dego} (Equation 6.1). Oligonucleotide binds to an antibody dendrimer conjugate (*Ab-D*) with a capacity, x, to form the oligonucleotide-antibody dendrimer conjugate, *Ab-D-O*. The oligonucleotide-antibody dendrimer conjugate binds to the IL-2R at the cell surface (R_s), forming a complex, $Ab\text{-}D\text{-}O\text{-}R_s$. The complex is then internalized by the receptor. Once the complex, $Ab\text{-}D\text{-}O\text{-}R_i$, is localized inside the cell, the oligonucleotide is released. The proposed rate expressions in which a conjugate binds to the IL-2R and is internalized by the receptor, followed by the trafficking and release of IL-2R (R_i) and oligonucleotide in the endosome (O_i) are given in Equations 6.2-6.5. Internalized oligonucleotides may further route to the lysosome, resulting in degraded oligonucleotides, O_{degi} (Equation 6.6).

$$O \xrightarrow{k_{\deg o}} O_{\deg o} \tag{6.1}$$

$$Ab - D + xO \underset{k_{do}}{\overset{k_{ao}}{\rightleftharpoons}} Ab - D - O_x \qquad \text{K} \tag{6.2}$$

$$Ab - D - O_x + R_s \underset{k_r}{\overset{k_f}{\rightleftharpoons}} Ab - D - O_x - R_s \tag{6.3}$$

$$Ab - D - O_x - R_s \xrightarrow{k_i} Ab - D - O_x - R_i \tag{6.4}$$

$$Ab - D - O_x - R_i \xrightarrow{fast} Ab - D - O_x + R_i \underset{k_{ai}}{\overset{k_{di}}{\rightleftharpoons}} Ab - D + xO_i + R_i \tag{6.5}$$

$$O_i \xrightarrow{k_{\deg i}} O_{\deg i} \tag{6.6}$$

Binding of oligonucleotide to antibody dendrimer conjugate, which corresponds to Equation 6.2, can be described by an equilibrium binding reaction. The equilibrium binding affinity constant (K) and binding capacity, x, are defined in chapter IV.

The corresponding rates of Equations 6.1-6.6 are described by Equations 6.7-6.13 (see definitions in Table 6.1).

Table 6.1. Definitions and typical units of variables and parameters.

Symbol	Definition	Units
O	Oligonucleotide concentration	M
Ab-D	Antibody dendrimer conjugate concentration	M
Ab-D-O	Oligonucleotide-antibody dendrimer conjugate concentration	M
R_s	Number of cell surface receptors	#/cell
C_s	Number of complexes at the cell surface	#/cell
N_A	Avogadro's number	#/mole
V_c	Cell volume	L/cell
N_{Rsyn}	Rate of new receptor synthesis	#/cell min^{-1}
C_i	Number of internalized complexes	#/cell
O_i	Oligonucleotide concentration in the endosome	M
R_i	Number of free receptors in the endosome	#/cell
E_i	Protein concentration inside the cell	mg/mL
O_{dego}	Degraded oligonucleotide concentration	M
O_{degi}	Degraded oligonucleotide concentration inside the cell	M
x	Amount of oligonucleotide per dendrimer	-
t	Localization time	min
k_{ao}	Association rate constant	M^{-1} min^{-1}
k_{do}	Dissociation rate constant	min^{-1}
K	Equilibrium binding affinity constant	M^{-1}
k_{dego}	Degradation rate constant	min^{-1}
k_f	Forward rate constant	M^{-1} min^{-1}
k_r	Reverse rate constant	min^{-1}
k_i	Internalization rate constant	min^{-1}
k_{ai}	Association rate constant inside the cell	M^{-1} min^{-1}
k_{di}	Dissociation rate constant inside the cell	min^{-1}
k_{degi}	Degradation rate constant inside the cell	$(mg/mL)^{-1}$ min^{-1}

$$\frac{dO}{dt} = -k_{ao}x[O][Ab-D] + k_{do}[Ab-D-O_x] - k_{\deg o}[O] \quad (6.7)$$

$$\frac{d(Ab-D)}{dt} = -k_{ao}x[O][Ab-D] + k_{do}[Ab-D-O_x] \quad (6.8)$$

$$\frac{d(Ab-D-O_x)}{dt} = k_{ao}x[O][Ab-D] - k_{do}[Ab-D-O_x] - \frac{k_f[Ab-D-O_x]R_s}{N_A V_c} + \frac{k_r C_s}{N_A V_c} \quad (6.9)$$

$$\frac{dR_s}{dt} = -k_f[Ab-D-O_x]R_s + k_r C_s + N_{Rsyn} \quad (6.10)$$

$$\frac{dC_s}{dt} = k_f[Ab-D-O_x]R_s - k_r C_s - k_i C_s \quad (6.11)$$

$$\frac{dC_i}{dt} = k_i C_s - k_{di} C_i \qquad k_{ai} << k_{di} \sim 0 \quad (6.12)$$

$$\frac{dO_i}{dt} = \frac{k_{di}xC_i}{N_A V_c} - k_{\deg i}E_i[O_i] \qquad k_{ai} << k_{di} \sim 0 \quad (6.13)$$

The parameters and estimates for the IL-2R model are listed in Table 6.2. A simulation using the model was performed using Polymath special educational version 5.1 software to find the amount of oligonucleotides in the endosome as a function of time. Results of the simulation are shown in Figures 6.1 and 6.2.

In comparison of simulation results for oligonucleotide internalized for the different dendrimers, total predicted oligonucleotide inside the cell without intracellular degradation correlates with binding of oligonucleotide to dendrimer conjugate. We had shown in earlier chapters that the binding capacity of the oligonucleotide for dendrimer was much less of generation 2.0 than generation 3.0 or 4.0. As seen in Figure 6.2C, oligonucleotide delivery is more effective when the generation 4.0 dendrimer was used.

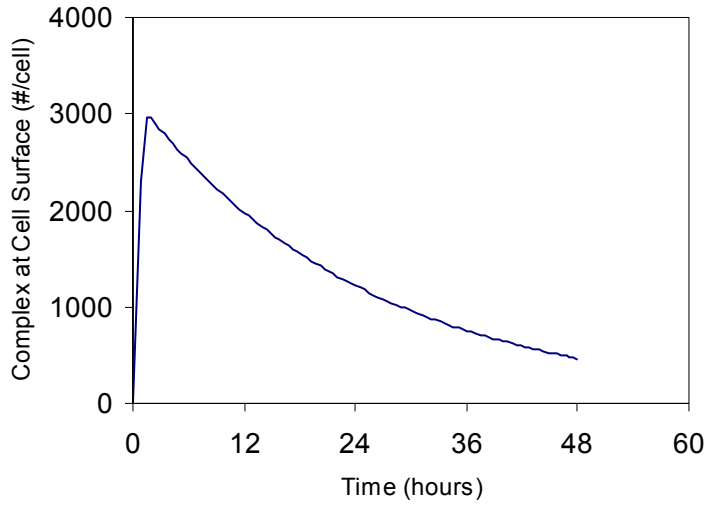
In examination of the effect of intracellular degradation of oligonucleotides, it is apparent that the effect of degradation is dramatic with all dendrimers tested. Based on simulation results, the rate of intracellular degradation of oligonucleotides plays an important role in our method. These data point to the importance of the delivery vehicle protecting oligonucleotide stability intracellularly.

Table 6.2. Parameters and estimates for the IL-2 receptor model.

Parameter	Value	Units	Reference
O	$3.0 \cdot 10^{-5}$	M	
Ab-D	$1.0 \cdot 10^{-8}$	M	
R_s	690	#/cell	Personal communication with Pacheco JR
N_{Rsyn}	29 ± 2	#/cell min^{-1}	Linares, 2000
N_A	$6.02 \cdot 10^{23}$	#/mole	
V_c^*	$5.2 \cdot 10^{-13}$	L/cell	Chapter III
E_i^*	10.9	mg/mL	Chapter III
$x_{G2.0}$	0.009 ± 0.002	-	Chapter IV
$x_{G3.0}$	0.46 ± 0.02	-	Chapter IV
$x_{G4.0}$	1.24 ± 0.05	-	Chapter IV
k_{ao}	$1.0 \cdot 10^{10}$	M^{-1} min^{-1}	
$K_{G2.0}$	$2.1 \pm 0.8 \cdot 10^{7}$	M^{-1}	Chapter IV
$K_{G3.0}$	$1.2 \pm 0.1 \cdot 10^{7}$	M^{-1}	Chapter IV
$K_{G4.0}$	$8.4 \pm 0.9 \cdot 10^{6}$	M^{-1}	Chapter IV
k_{dego} (conditioned)	0.000031	min^{-1}	Chapter III
k_f	$1.8 \cdot 10^{9}$	M^{-1} min^{-1}	Linares, 2000
k_r	0.013	min^{-1}	Linares, 2000
$k_{iG2.0}$	0.00083	min^{-1}	Chapter V
$k_{iG3.0}$	0.00067	min^{-1}	Chapter V
$k_{iG4.0}$	0.00033	min^{-1}	Chapter V
$k_{diG2.0}$	476	min^{-1}	
$k_{diG3.0}$	833	min^{-1}	
$k_{diG4.0}$	1190	min^{-1}	
k_{degi} (cell lysate)	0.0121 ± 0.0011 or 0.0002	$(mg/mL)^{-1}$ hr^{-1} $(mg/mL)^{-1}$ min^{-1}	Chapter III

* is based on the cell with an approximate size of 10 μm.

A



B

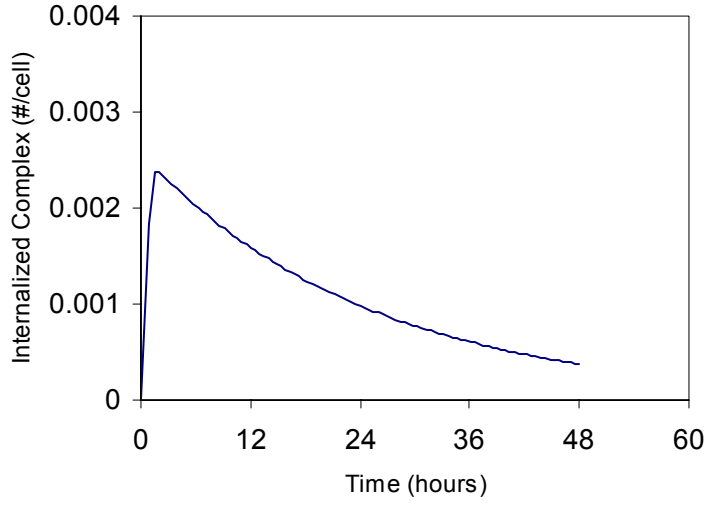


Figure 6.1. Model simulation of oligonucleotide delivery using generation 3.0 dendrimer as the delivery vehicle. The simulation using the model was performed in the presence of intracellular degradation. (A) Complex at the cell surface is plotted as number per cell *versus* time. (B) Internalized complex is plotted as number per cell *versus* time. (C) Internalized oligonucleotide is plotted as number per cell *versus* time.

C

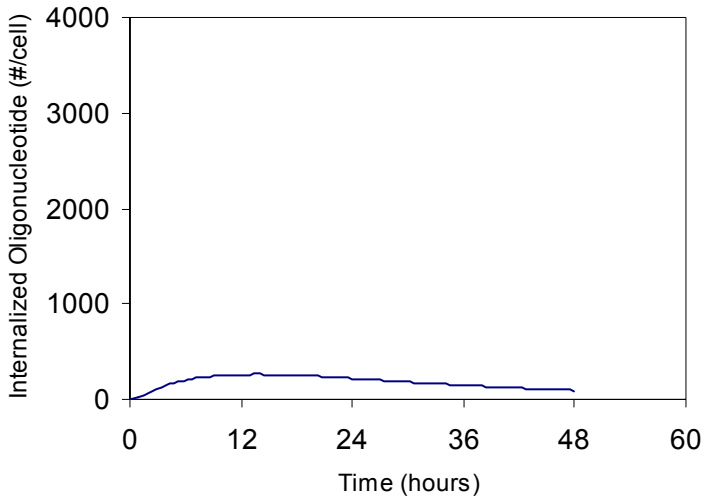
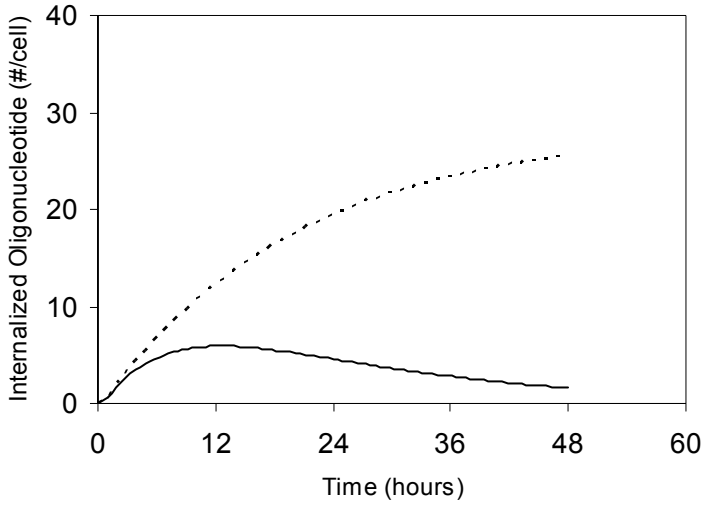


Figure 6.1. (Continued)

A



B

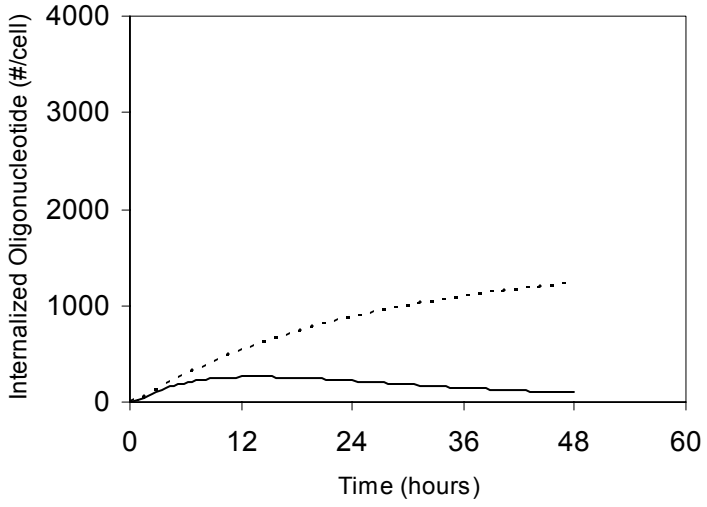


Figure 6.2. Model simulation of role of intracellular degradation. The simulation using the model was performed in the presence (—) and absence (---) of intracellular degradation. Internalized oligonucleotide is plotted as number per cell *versus* time. (A) Generation 2.0. (B) Generation 3.0. (C) Generation 4.0.

C

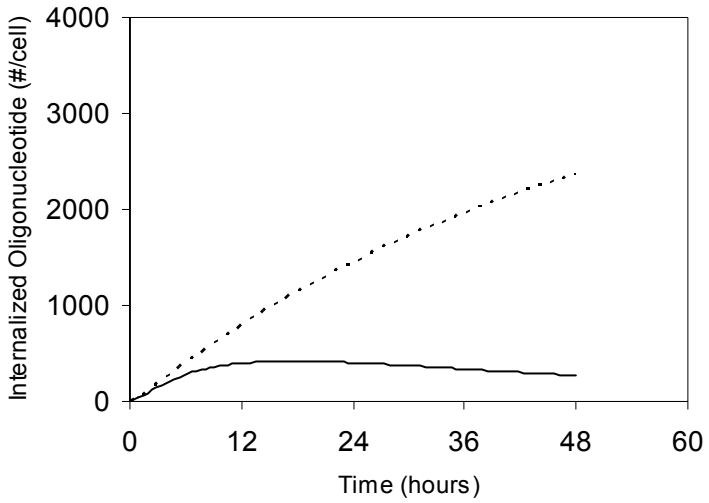


Figure 6.2. (Continued)

CHAPTER VII
CONCLUSIONS AND RECOMMENDATIONS

The effectiveness of most chemotherapeutic regimens is limited by the toxicity of the therapy to normal healthy cells. Therapies to selectively modulate abnormal T cells bearing the IL-2R have been developed to treat diseases associated with immune disorders. The goal of the research described in this work was to develop the engineering or rational design of agents which deliver oligonucleotides to specific T cells to selectively and effectively modulate immune response; and to examine the steps and key components in the mechanism underlying oligonucleotide delivery.

CONCLUSIONS

The expression of the IL-2R α chain on some abnormal cells provides us with a target and the opportunity to selectively destroy these cells. Such strategies have been the focus of therapeutic intervention associated with lymphoid malignancies, select autoimmune diseases, GVHD, and allograft rejection. Oligonucleotide therapeutic approaches are designed to introduce genetic materials into abnormal cells to modulate the expression of a target protein. Steps involved in oligonucleotide transfer include cellular uptake, intracellular trafficking, and (if necessary) nuclear translocation. The aim of this body of research was, taking advantages of engineering approaches, (1) to investigate the specificity and efficacy of oligonucleotide delivery using a monoclonal antibody for targeting and a positively charged PAMAM dendrimer as the carrier for the oligonucleotide to the IL-2R bearing T cells; (2) to examine the relative stability of oligonucleotides in a cellular environment; and (3) to explore the properties including toxicity of the dendrimer delivery vehicle, the binding capacity of the vehicle, affinity of the vehicle for oligonucleotides, and cellular uptake of the vehicle for oligonucleotide delivery.

First, we examined the effect of oligonucleotides with different backbone chemistries on cell viability in the presence and absence of dendrimer delivery vehicle.

In all cases, the oligonucleotides had relatively low toxicity in the model leukemia cell line used in our experiments.

We describe the development of an HPLC method to examine the relative stability of oligonucleotides against intracellular and extracellular nucleases. Using a size exclusion HPLC technique, we were able to determine the portions of the oligonucleotide based on the difference in size and/or molecular mass that were degraded during incubation. Data from the degradation assay and parameter estimation suggest that the rate of intracellular degradation was much greater than extracellular degradation.

To further protect oligonucleotides from intracellular degradation, the oligonucleotide was complexed to the dendrimer. We developed a cationic-exchange HPLC method using a linear salt gradient to separate the oligonucleotide and the dendrimer. Our results showed that almost 100% of the oligonucleotide was separated from the dendrimer complex. It should be noted that considerable aggregation of discrete particles was observed while incubating the complexes in cell lysate. We further examined this aggregation phenomenon. Several attempts had been made in order to dissolve the pellets. However, we were unable to dissolve these resulting aggregates, suggesting that the complexes and cell lysate may have an inherent tendency toward aggregation under physiological buffer conditions.

An investigation of the properties of the dendrimer vehicles, toxicity, binding capacity and binding affinity, as a function of dendrimer generation, was carried out. We conducted an equilibrium binding analysis as well as estimation of equilibrium binding constant of oligonucleotides to dendrimers. The binding capacity of the same dendrimer was also examined as a function of the ionic strength of the buffers. Our results address the importance of the complex formation between the delivered oligonucleotide and a carrier, which may aid in the design of a more effective therapeutic vehicle for oligonucleotide delivery.

The kinetic and thermodynamic analysis of a delivery vehicle of an antibody dendrimer conjugate was performed as a function of dendrimer generation. Vehicle

delivery involves binding of the vehicle to the receptor and internalization of the receptor-vehicle complex. According to our results, the dendrimer conjugate of a certain size and surface charge, which has less tendency to aggregate, is required for receptor-mediated oligonucleotide delivery. More importantly, the internalization plays a pivotal role as a rate limiting step in the mechanism of oligonucleotide delivery.

Finally, a mathematical model was developed to study the IL-2R targeted gene therapy. *In vitro* data are incorporated into derived rate equations to predict the performance. This part of the work demonstrates the utility of an engineering approach to examining therapeutic parameters based on the targeting of the IL-2R, and could provide insight into the design and optimization of immunomodulatory therapy.

In summary, this body of research explores the engineering or rational design of targeted oligonucleotide delivery vehicles selectively to T cells expressing the IL-2R. The ability to predict the effects of different delivery vehicle properties on oligonucleotide delivery may aid in the development of design criteria for new vehicles for delivery of antisense, siRNA, or genes to IL-2R bearing cells.

RECOMMENDATIONS

Validation of the Biological Effect of PDE1B1 mRNA

Our results showed that the oligonucleotides fail to significantly affect cell viability. To better measure its biological effect, studies would need to examine if the oligonucleotide is able to lead to reduced levels of cellular mRNA of the gene of interest, and to lead to attenuated protein levels, independent of cell survival. The results from western blotting along with DNA fragmentation analysis might give complete information on the biological response to oligonucleotide. We believe these results would enable us to better explain our previous data.

Identification of Genes in Jurkat Cells

Our results showed that the oligonucleotides had relatively low toxicity effect. To select more effective sequences, it would be useful to obtain more details of relevant

gene targets. The polymerase chain reaction along with direct DNA sequencing might be useful for amplifying genes encoding proteins in Jurkat leukemia T cells.

Interaction between the Dendrimer Complex and Cell Lysate

We were unable to characterize to our satisfaction the effect of dendrimer on rate of degradation of oligonucleotide intracellularly, in part because the dendrimer-oligonucleotide complex aggregated in cell lysate. In examining the effect on the aggregation of the dendrimer complexes in cell lysate, several suitable non-charged detergents need to be found and used to dissolve the pellets. In addition, an antibody dendrimer conjugate with altered affinity might be used in experiments to examine the effect of the complex in oligonucleotide degradation experiments.

Optimization of IL-2 Receptor Targeted Gene Delivery Vehicle

Optimization of the delivery vehicle has proven to be critical to enhance the rate of internalization of oligonucleotide. Some evidence revealed that the fractured dendrimer increased transfection efficiency due to its increased degree of flexibility. In the future, we might explore the effect of fractured dendrimers in internalization, binding, and stability experiments to further optimize our delivery vehicle.

Specificity of Oligonucleotide Delivery

The original goal of this work was to demonstrate that we could specifically and effectively deliver an oligonucleotide to cells which would affect cell cycle and result in reduction in proliferation or death of a specific population of cancerous T cells. While we were able to deliver oligonucleotide to T cells, we were never able to effectively kill those cells with the antisense and small interfering RNA's chosen. Therefore, we never tested the specificity of the therapy. Once an appropriate oligonucleotide is found that will actually reduce proliferation of cells, it would be useful then to test the vehicle and oligonucleotide against irrelevant cell lines.

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APPENDIX

MEASUREMENT OF OLIGONUCLEOTIDE BINDING TO DENDRIMERS

Conjugation of Dendrimers to Gels

The H_2N-terminated and HOOC-terminated dendrimers were coupled to aminolink and immobilized DADPA gels, respectively, according to the manufacturer's instruction. Table A.1 summarizes the conjugation efficiency for coupling of dendrimers to gels.

Estimation of Nonspecific Binding of Oligonucleotides to Quenched Gels

The total binding can be expressed as the simple sum of specific and nonspecific binding (Equation A.1).

$$[B_T] = [B_S] + [B_{NS}] \quad \text{(A.1)}$$

For an aminolink gel, the nonspecific binding of oligonucleotide to the gel was negligible, while for an immobilized DADPA gel, isotherms were corrected for nonspecific binding. If we can assume that the nonspecific binding is a linear function of the oligonucleotide concentration, then the observed binding will be described as:

$$[B_T] = [B_S] + aV_{gel}[F] \quad \text{(A.2)}$$

where a is a constant and V_{gel} represents the volume of quenched gel. A linear least squares regression analysis of binding of oligonucleotides to the quenched DADPA gel (Figure A.1) is given a constant of 0.0953 μL^{-1}.

Table A.1. Summary of conjugation efficiency for coupling of dendrimers to gels.

Generation	**%Conjugation efficiency**
2.0	47.7
3.0	52.1
3.5*	39.8
4.0	75.9
4.5	34.5

* indicates 2-fold mole loading of dendrimer in the coupling reaction compared with other dendrimers.

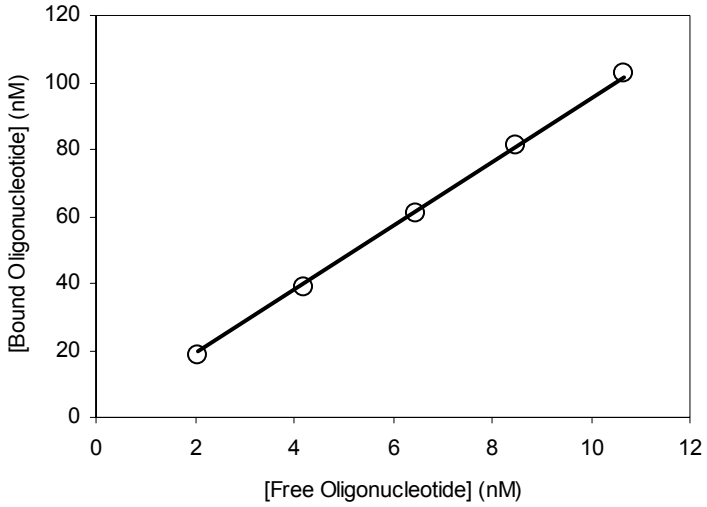

Figure A.1. Nonspecific binding of FITC-labeled oligonucleotides to quenched DADPA gel. Experiments were conducted at room temperature in phosphate buffer saline (PBS, 13.67 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2 PO_4) at pH 7.2. The amount of oligonucleotide bound to the quenched gel is plotted *versus* concentration of free oligonucleotide.

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